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(54) Title: CONFORMATIONALLY CONSTRAINED BACKBONE CYCLIZED SOMATOSTATIN ANALOGS		
(57) Abstract Novel peptide analogs are disclosed. The novel peptides are conformationally constrained backbone cyclized somatostatin analogs. Methods for synthesizing the somatostatin analogs and for producing libraries of the somatostatin analogs are also disclosed. Furthermore, pharmaceutical compositions comprising somatostatin analogs, and methods of using such compositions in the treatment of endocrine disorders, neoplasms and metabolic disorders are also disclosed.		

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**CONFORMATIONALLY CONSTRAINED BACKBONE
CYCLIZED SOMATOSTATIN ANALOGS**

FIELD OF THE INVENTION

5 The present invention relates to conformationally
constrained N^α backbone-cyclized somatostatin analogs cyclized
via novel linkages, to processes for the preparation of these
backbone cyclized peptide analogs, to methods for using these
peptide analogs and to pharmaceutical compositions containing
10 same.

BACKGROUND OF THE INVENTION

Somatostatin analogs

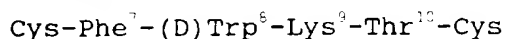
15 Somatostatin is a cyclic tetradecapeptide found both in
the central nervous system and in peripheral tissues. It was
originally isolated from mammalian hypothalamus and
identified as an important inhibitor of growth hormone
secretion from the anterior pituitary. Its multiple
biological activities include inhibition of the secretion of
20 glucagon and insulin from the pancreas, regulation of most
gut hormones and regulation of the release of other
neurotransmitters involved in motor activity and cognitive
processes throughout the central nervous system (for review
see Lamberts, Endocrine Rev., 9:427, 1988). Additionally,
25 somatostatin and its analogs are potentially useful
antiproliferative agents for the treatment of various types
of tumors.

Natural somatostatin (also known as Somatotropin Release
Inhibiting Factor, SRIF) of the following structure:
30 H-Ala¹-Gly²-Cys³-Lys⁴-Asn⁵-Phe⁶-Phe⁷-Trp⁸-Lys⁹-Thr¹⁰-Phe¹¹-Thr¹²-
Ser¹³-Cys¹⁴-OH
was first isolated by Guillemin and colleagues (Bruzeau et
al. Science, 179:78, 1973). It exerts its effect by
interacting with a family of receptors. Recently five

properties: poor bioavailability and short duration of action. For this reason, great efforts have been made during the last two decades to find somatostatin analogs that will have superiority in either potency, biostability, duration of
 5 action or selectivity with regard to inhibition of the release of growth hormone, insulin or glucagon.

Structure-activity relation studies, spectroscopic techniques such as circular dichroism and nuclear magnetic resonance, and molecular modeling approaches reveal the
 10 following: the conformation of the cyclic part of natural somatostatin is most likely to be an antiparallel β -sheet; Phe⁶ and Phe¹¹ play an important role in stabilizing the pharmacophore conformation through hydrophobic interactions between the two aromatic rings; the four amino acids Phe⁷-
 15 Trp⁹-Lys⁹-Thr¹⁰ which are spread around the β -turn in the antiparallel β -sheet are essential for the pharmacophore; and (D)Trp⁸ is preferable to (L)Trp⁸ for the interactions with somatostatin receptor subtypes 2 through 5.

Nevertheless, a hexapeptide somatostatin analog
 20 containing these four amino acids anchored by a disulfide bridge:



25 is almost inactive both *in vitro* and *in vivo*, although it has the advantage of the covalent disulfide bridge which replaces the Phe⁶-Phe¹¹ hydrophobic interactions in natural somatostatin.

Four main approaches have been attempted in order to
 30 increase the activity of this hexapeptide somatostatin analog. (1) Replacing the disulfide bridge by a cyclization which encourages a cis-amide bond, or by performing a second cyclization to the molecule yielding a bicyclic analog. In both cases the resultant analog has a reduced number of

other natural or non-natural amino acids, such as replacing

Phe¹ with Tyr¹ and Thr¹⁰ with Val¹⁰. (3) Incorporating additional functional groups from natural somatostatin with the intention that these new elements will contribute to the interaction with the receptor. (4) Eliminating one of the four amino acids Phe⁷-(D)Trp⁸-Lys⁹-Thr¹⁰ with the assumption that such analogs would be more selective.

The somatostatin analog, MK-678:

cyclo(N-Me-Ala⁶-Tyr⁷-(D)Trp⁸-Lys⁹-Val¹⁰-Phe)

10

is an example of a highly potent somatostatin analog designed using the first three approaches above (Veber, et al., Life Science, 34:371, 1984). In this hexapeptide analog, a cis-amide bond is located between N-Me-Ala and Phe¹¹, Tyr⁷ and Val¹⁰ replace Phe¹ and Thr¹⁰ respectively, and Phe¹¹ is incorporated from natural somatostatin.

Another group of somatostatin analogs (U.S. patents 4,310,518 and 4,235,886) includes Octreotide:

20 H-(D)Phe⁵-Cys-Phe⁷-(D)Trp⁸-Lys⁹-Thr¹⁰-Cys-Thr¹¹-CH₂OH

the only approved somatostatin analog currently available. It was developed using the third approach described above.

25 Here, (D)Phe⁵ and the reduced C-terminal Thr¹¹-CH₂OH are assumed to occupy some of the conformational space available to the natural Phe¹ and Thr¹⁰, respectively.

The compound TT-232:

30

H-(D)Phe-Cys-Phe⁷-(D)Trp⁸-Lys⁹-Cys-Thr-NH₂

is closely related to Octreotide and is an example of

These examples of highly potent somatostatin analogs suggest that the phenylalanines in positions 6 and 11 not only play an important role in stabilizing the pharmacophore conformation but also have a functional role in the
5 interaction with the receptor. It is still an open question whether one phenylalanine (either Phe⁶ or Phe¹¹) is sufficient for the interaction with the receptor or whether both are needed.

It is now known that the somatostatin receptors
10 constitute a family of five different receptor subtypes (Bell and Reisine, Trends Neurosci., 16, 34-38, 1993), which may be distinguished on the basis of their tissue specificity and/or biological activity. Somatostatin analogs known in the art may not provide sufficient selectivity or receptor subtype
15 selectivity, particularly as anti-neoplastic agents (Reubi and Laissue, TIPS, 16, 110-115, 1995).

Symptoms associated with metastatic carcinoid tumors (flushing and diarrhea) and vasoactive intestinal peptide (VIP) secreting adenomas (watery diarrhea) are treated with
20 somatostatin analogs. Somatostatin has been also approved for the treatment of severe gastrointestinal hemorrhages. Somatostatin may also be useful in the palliative treatment of other hormone-secreting tumors (e.g., pancreatic islet-cell tumors and acromegaly) and hormone dependent tumors
25 (e.g., chondrosarcoma and osteosarcoma) due to its antisecretory activity.

Peptidomimetics

As a result of major advances in organic chemistry and
30 in molecular biology, many bioactive peptides can now be prepared in quantities sufficient for pharmacological and clinical utilities. Thus in the last few years new methods have been established for the treatment and therapy of illnesses in which peptides have been implicated. However,

The international patent application, PCT/IL97/00261, is hereby incorporated by reference into this document.

absorption after oral ingestion, in particular due to their relatively high molecular mass or the lack of specific transport systems or both; c) their rapid excretion through the liver and kidneys; and d) their undesired side effects in non-target organ systems, since peptide receptors can be widely distributed in an organism.

Moreover, with few exceptions, native peptides of small to medium size (less than 30 amino acids) exist unordered in dilute aqueous solution in a multitude of conformations in dynamic equilibrium which may lead to lack of receptor selectivity, metabolic susceptibilities and hamper attempts to determine the biologically active conformation. If a peptide has the biologically active conformation per se, i.e., receptor-bound conformation, then an increased affinity toward the receptor is expected, since the decrease in entropy on binding is less than that on the binding of a flexible peptide. It is therefore important to strive for and develop ordered, uniform and biologically active peptides.

In recent years, intensive efforts have been made to develop peptidomimetics or peptide analogs that display more favorable pharmacological properties than their prototype native peptides. The native peptide itself, the pharmacological properties of which have been optimized, generally serves as a lead for the development of these peptidomimetics. However, a major problem in the development of such agents lies in determining the active region of a biologically active peptide. For instance, frequently only a small number of amino acids (usually four to eight) are responsible for the recognition of a peptide ligand by a receptor. Once this biologically active site is determined a lead structure for development of peptidomimetic can be optimized, for example by structure-activity relationship studies.

interacting with the receptor. The active site of a peptide at the

receptor level. The following factors should be considered to achieve the best possible agonist peptidomimetic a) metabolic stability, b) good bioavailability, c) high receptor affinity and receptor selectivity, and d) minimal
5 side effects.

A generally applicable and successful method recently has been the development of conformationally restricted peptidomimetics that imitate the receptor-bound conformation of the endogenous peptide ligands as closely as possible
10 (Rizo and Gierasch, Ann. Rev. Biochem., 61:387, 1992). Investigations of these types of analogs show them to have increased resistance toward proteases, that is, an increase in metabolic stability, as well as increased selectivity and thereby fewer side effects (Veber and Friedinger, Trends
15 Neurosci., p. 392, 1985).

Once these peptidomimetic compounds with rigid conformations are produced, the most active structures are selected by studying the structure-activity relationships. Such conformational constraints can involve local
20 modifications of structure or global conformational restraints (for review see Giannis and Kolter, Angew. Chem. Int. Ed. Engl. 32:1244, 1993).

Conformationally constrained peptide analogs

25 Bridging between two neighboring amino acids in a peptide leads to a local conformational modification, the flexibility of which is limited in comparison with that of regular dipeptides. Some possibilities for forming such bridges include incorporation of lactams and piperazinones.
30 γ -Lactams and δ -lactams have been designed to some extent as "turn mimetics"; in several cases the incorporation of such structures into peptides leads to biologically active compounds.

Global restrictions in the conformation of a peptide are

... of ... peptides

The main limitations to these classical modes of cyclization are that they require substitution of amino acid side chains in order to achieve cyclization.

In Gilon, EPO Application No. 564,739 A2; and J. Org. Chem., 57:5687, 1992, two basic approaches to the synthesis of building units are described. The first starts with the

chemistry peptide synthesis. The second approach starts with selective protection of a diamine and reaction of the product with chloroacetic acid to provide the protected glycine derivative, suitable for Fmoc peptide synthesis.

- 5 Further modes of utilizing the concept of backbone cyclized peptide analogs are disclosed in WO95/33765 which also provides novel methods of synthesizing building units other than glycine. Among the families of peptide analogs disclosed in WO95/33765 are various somatostatin analogs.
- 10 None of the families of analogs disclosed in that application is shown to have unique attributes or unexpected advantages in terms of the binding to receptor subtypes.

Libraries of backbone cyclized peptide analogs

- 15 As mentioned above linear peptides suffer from several serious drawbacks as potential drugs, inasmuch as they are notoriously unstable in vivo, often lack high affinity of binding to their receptor, frequently lack selectively to one kind of receptor, and generally have poor oral
- 20 bioavailability. In efforts to overcome such problems, it is also possible to utilize the methodologies developed in connection with synthetic peptide libraries to generate collections of cyclic peptides, novel biopolymers and even novel branched oligomeric compounds (reviewed by Zuckermann,
- 25 Current Opinion in Structural Biology 3, 580-584, 1993).

The generation of libraries of cyclic peptides requires that in addition to any previously stated considerations, the cyclization reaction be performed in a high yield and with a minimum of additional manipulations. Unfortunately,

30 classical cyclization reactions are highly sequence dependent in terms of the expected yields, making the uniform cyclization of a peptide mixture unreliable.

Recent advances in the cyclization of peptides directly on the solid support have improved the synthetic procedure,

35

conditions of high dilution. Polymer-supported cyclizations can both avoid potential side reactions such as oligomerization and facilitate product purification. For example, on-resin cyclization methods have recently been used
5 to prepare cyclopeptides with bridges formed of thioethers, disulfides, or lactams between two side chains, lactam between the amino terminus and a side chain, and lactams between the amino and carboxy termini (reviewed by Zuckermann, Current Opinion in Structural Biology 3, *ibid*).

10 The use of resin-bound cyclic peptides and free cyclic peptides in combinatorial libraries is disclosed in WO 92/00091. However, these cyclic peptides do not contain any conformationally constraining element, and in cases where cyclization is achieved, these peptides may still adopt a
15 number of conformations and suffer many of the same shortcomings as linear peptides.

Cyclic semi-random peptide libraries, disclosed in WO 95/01800, are exclusively cyclic penta- and hexa-peptide libraries containing one or more randomized amino acids and a
20 conformationally constraining element in the form of an amino acid residue such as proline which fixes the beta turn angles of the adjacent amino acid residues. The advantages of such conformationally constraining elements is stressed by the inventors of this approach. However, inclusion of such
25 elements via incorporation of a particular amino acid residue into the peptide sequence may have detrimental effects on those residues required for receptor recognition or other biological activity. Furthermore, in that disclosure (WO 95/01800), the cyclization reaction is merely another
30 coupling reaction in which the terminal amino group of the linear peptide is coupled to the terminal carboxy group of the peptide.

SUMMARY OF THE INVENTION

According to the present invention, novel peptidomimetic compounds, which are characterized in that they incorporate novel building units with bridging groups attached to the
5 alpha nitrogens of alpha amino acids; have now been generated.

The most striking advantages of this approach are:

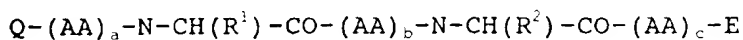
- 1) The method enables cyclization of the peptide sequence without compromising any of the side chains of the peptide
10 thereby decreasing the chances of sacrificing functional groups essential for biological recognition and function.
- 2) The method allows optimization of the peptide conformation by allowing permutation of the bridge length, direction, and bond type (e.g., amide, disulfide, thioether, thioester,
15 etc.) and position of the bond in the ring. 3) When applied to cyclization of linear peptides of known activity, the bridge can be designed in such a way as to minimize interaction with the active region of the peptide and its cognate receptor. This decreases the chances of the
20 cyclization arm interfering with recognition and function, and also creates a site suitable for attachment of tags such as radioactive tracers, cytotoxic drugs, light capturing substances, or any other desired label.

The newly generated libraries, disclosed according to
25 the present invention, now enable varying conformation as well as level of flexibility (constraint) in order to find the optimal backbone conformation of the peptide in performing its role as an agonist or antagonist. This is accomplished by varying both the position of the bridgeheads
30 (i.e., the positions in the linear sequence of residues that are to be cyclized), as well as varying the length, the direction and the bond type of the bridge between these units.

It is another object of the present invention to provide

FIGURE 1 is a diagram of a peptide sequence with a bridgehead group, as described below.

In the present invention, one or more pairs of the building units is joined together to form a cyclic structure. Thus, according to one aspect of the present invention, backbone cyclized somatostatin analogs are provided that have the
 5 general Formula (I):



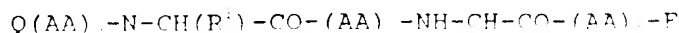
Formula (I)

10

wherein: a - c each independently designates an integer from 1 to 8 or zero; (AA) designates an amino acid residue wherein the amino acid residues in each chain may be the same or different; Q represents H or an acyl group; E represents a
 15 hydroxyl group, a carboxyl protecting group or an amino group, or the terminal carboxyl group can be reduced to CH₂-OH; R¹ and R² each designates an amino acid side-chain optionally bound with a specific protecting group; and the line designates a bridging group of the Formula:
 20 (i) -X-M-Y-W-Z- ; or (ii) -X-M-Z-wherein: M and W are independently selected from the group consisting of amide, thioether, thioester and disulfide; and X, Y and Z are each independently selected from the group consisting of alkylene, substituted alkylene, arylene, homo- or hetero- cycloalkylene
 25 and substituted cycloalkylene.

In certain preferred embodiments, the terminal carboxyl group of Formula (I) is replaced by a carboxy terminal amide or is reduced to a CH₂OH group.

Another embodiment of the present invention involves
 30 N-backbone to side chain cyclized somatostatin analogs of the general formula (II):



wherein: d - f each independently designates an integer from 1 to 8 or zero; (AA) designates an amino acid residue wherein the amino acid residues in each chain may be the same or different; Q represents H or an acyl group; E represents a hydroxyl group, a carboxyl protecting group or an amino group, or the terminal carboxyl group can be reduced to $\text{CH}_2\text{-OH}$; R^1 designates an amino acid side-chain optionally bound with a specific protecting group; and the line designates a bridging group of the Formula: (i) $-\text{X-M-Y-W-Z}-$; or

10 (ii) $-\text{X-M-Z}-$ wherein M and W are independently selected from the group consisting of amide, thioether, thioester and disulfide; and X, Y and Z are each independently selected from the group consisting of alkylene, substituted alkylene, arylene, homo- or hetero- cycloalkylene and substituted

15 cycloalkylene.

A preferred embodiment of the present invention involves the backbone cyclized somatostatin analog of Formulae I or II wherein the line designates a bridging group of the Formula $-(\text{CH}_2)_x-\text{M}-(\text{CH}_2)_y-$; M is selected from the group consisting of

20 amide, thioether, thioester and disulfide; and x and y each independently designates an integer from 1 to 10.

Further preferred are backbone cyclized somatostatin analogs of the Formula I or II wherein R^1 and R^2 are other than H, such as CH_3 , $(\text{CH}_3)_2\text{CH}-$, $(\text{CH}_3)_2\text{CHCH}_2-$, $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)-$,

25 $\text{CH}_3\text{S}(\text{CH}_2)_1-$, HOCH_2- , $\text{CH}_3\text{CH}(\text{OH})-$, HSCH_2- , $\text{NH}_2\text{C}(=\text{O})\text{CH}_2-$, $\text{NH}_2\text{C}(=\text{O})(\text{CH}_2)_1-$, $\text{NH}(\text{CH}_2)_1-$, $\text{HOC}(=\text{O})\text{CH}_2-$, $\text{HOC}(=\text{O})(\text{CH}_2)_1-$, $\text{NH}_2(\text{CH}_2)_4-$, $\text{C}(\text{NH}_2)\text{NH}(\text{CH}_2)_3-$, HO-phenyl- CH_2- , benzyl, methylindole, and methylimidazole.

Another preferred aspect of the present invention is

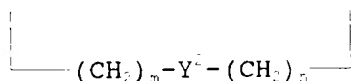
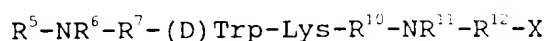
30 directed to backbone cyclization to generate novel somatostatin analogs linked between positions 6 and 11, leaving the phenylalanine side chains untouched. This conformational stabilization is much more rigid than the Phe^1 , Phe^{27} hydrophobic interaction in natural somatostatin and is

stable covalent bridge can be achieved while either one or both of the original Phe¹ and Phe¹¹ are retained.

Moreover, backbone cyclizations can also be used to anchor the β -turn, not only in positions 6 and 11 but also
 5 inside the active reaction of Phe⁷-(D)Trp⁸-Lys⁹-Thr¹⁰, yielding either a monocyclic analog with a preferable conformation or a very rigid bicyclic analog. Here again, the side chains of the pharmacologically active amino acids remain untouched and the only change is in limiting the conformational space.

10 As used herein and in the claims in the following more preferred backbone cyclized peptide analogs, the superscript numbers following the amino acids refer to their position numbers in the native Somatostatin.

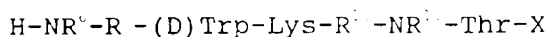
A more preferred backbone cyclized somatostatin analog
 15 is the Formula (Va):



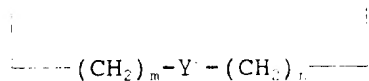
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Formula (Va)

a most preferred analog having Formula (Vb):



25

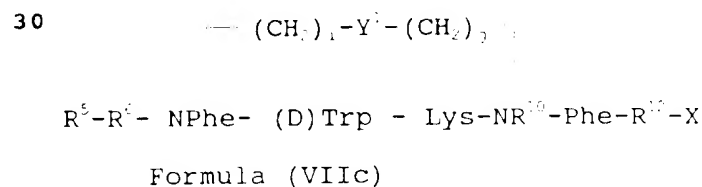
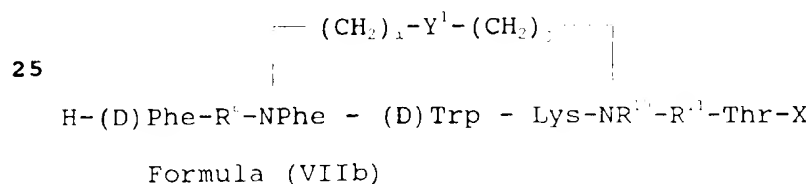
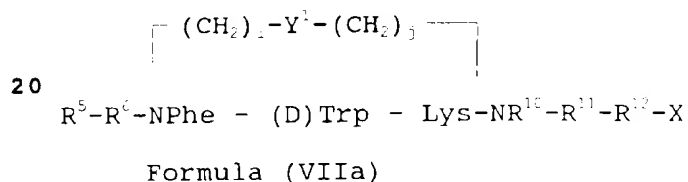
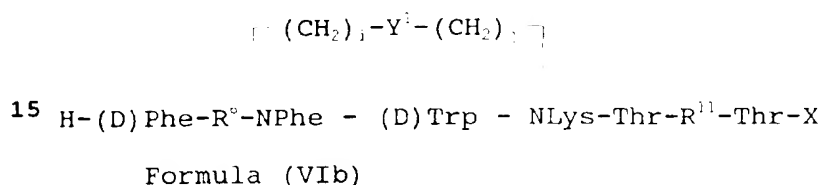
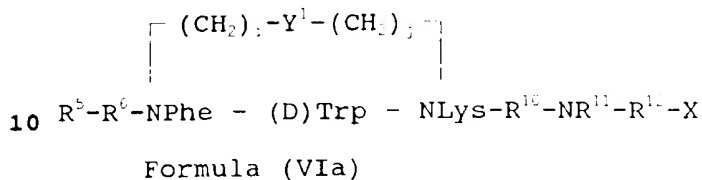


Formula (Vb)

wherein m and n are 1 to 5; X designates a carboxy terminal
 30 amide or alcohol; R⁶ is absent or is Gly, (D)- or (L)-Ala, (D)- or (L)-Phe, Nal and β -Asp(Ind); R⁵ and R¹¹ are independently Gly or (D)- or (L)-Phe; R⁷ is Phe or Tyr; R¹⁰ is absent or is Gly, Abu, Thr or Val; R¹² is absent or is Val, Thr or Nal, and Y is selected from the group consisting of

phenylalanine side chains as in the natural somatostatin. Still more preferred is the analog wherein Phe⁷ is replaced with Tyr⁷ and Thr¹⁰ is replaced with Val¹⁰.

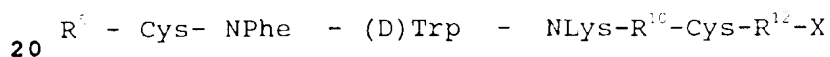
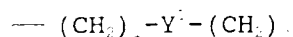
Other preferred monocyclic analogs that anchor the molecule in positions inside the active region rather than in positions 6 and 11 are formulae VI (a and b) and VII (a-c):



wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; Rⁱ is absent or is (D) - or (L)-Phe, Nal, or β-Asp(Ind); R^j is (D) or (L) -Phe; R¹ⁱ is absent or is Gly, Abu or Thr; and R^{1j} is (D)- or (L)-Phe; R¹ⁱ is absent or is Thr or Nal, and Yⁱ is selected from the group consisting of amide, thioether, thioester and disulfide.

Still other preferred analogs incorporate backbone cyclization in positions 6 and 11 as in Formula V, together with the backbone cyclizations as in Formula VI and VII, yielding rigid bicyclic analogs.

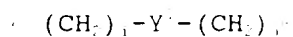
Other more preferred bicyclic analogs differ from Formulae V-VII by the replacement of the amino acids at positions 6 and 11 by cysteines which form a disulfide bond, leaving only one backbone cyclization in the Formulae VIII(a and b) and IX (a and b):

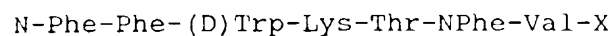
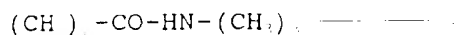


Formula (VIIIa),

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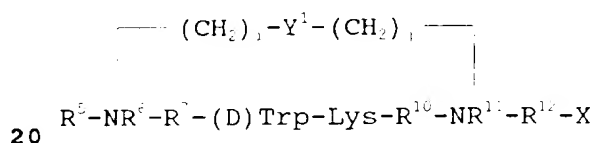


5 (i.e., cyclo [NPhe-Phe-(D)Trp-Lys-Thr-NPhe]-Val-X:
denoted PTR 3040)

wherein X denotes a carboxy terminal acid, amide, ester or alcohol. These two analogs have been shown to possess unexpectedly useful properties due to their selectivity to particular somatostatin receptor subtypes.

10 The more preferred monocyclic somatostatin analogs may also be prepared as libraries of active analogs, that are particularly useful to screen for the optimal conformers.

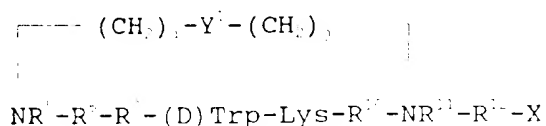
Yet more preferred somatostatin analogs according to the present invention include compositions of the formulae X
15 through XIV:



Formula X

wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R^5 is (D)Phe or 2-Nal; R^5
25 is Phe, Gly or Ala; R^i is Tyr or pClPhe; R^{10} is Thr, Val, Ser or Abu; R^{11} is Phe, Gly or Ala; R^{12} is Thr, Val, 2-Nal or (D)2-Nal, and Y^1 is selected from the group consisting of amide, thioether, thioester and disulfide.

30 Formula XI

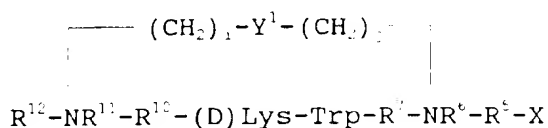


wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R^i is (D)Phe or 2-Nal; R^j is Phe, Gly or Ala; R^k is Tyr or pClPhe; R^{10} is Thr, Val, Ser or Abu; R^{11} is Phe, Gly or Ala; R^{12} is Thr, Val, 2-Nal or (D)2-Nal, and Y^2 is selected from the group consisting of amide, thioether, thioester and disulfide.

or Lys; R^1 is absent or is Phe; R^2 is Tyr or Phe; R^3 is absent or Thr, Val, Ser or Abu; R^4 is Phe, Gly or Ala; R^5 is Trp, Thr, Val, 2-Nal or (D)2-Nal, and Y^1 is selected from the group consisting of amide, thioether, thioester and disulfide.

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Formula XII



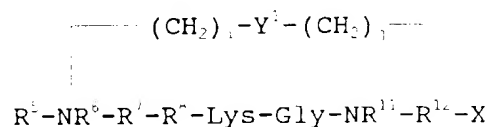
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wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R^5 Phe, (L)2-Nal or (D)2-Nal; R^6 is Phe, Gly or Ala; R^7 is (D)Phe, pCl(D)Phe, pNH₂Phe or (D)Tyr; R^{10} is (D)Thr, (D)Val (D)Ala, (D)Leu or (D)Glu; R^{11} is Phe, Gly or Ala; R^{12} is absent or is Thr or Val; and Y^1 is selected from the group consisting of amide, thioether, thioester and disulfide.

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Formula XIII

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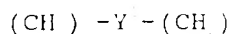


wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R^1 is absent or (D)Phe or 2-Nal; R^2 is Phe, Gly or Ala; R^3 is (D)Phe, pCl(D)Phe, pNH₂Phe or (D)Tyr; R^4 is (D) or (L)Trp; R^{10} is (D)Thr, (D)Val, (D)Ala, (D)Leu or (D)Glu; R^{11} is Phe, Gly or Ala; R^{12} is Thr, Val, Ala, β -Ala, (L)2-Nal or (D)2-Nal; and Y^2 is selected from the group consisting of amide, thioether, thioester and disulfide.

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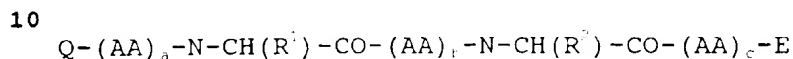
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Formula XIV



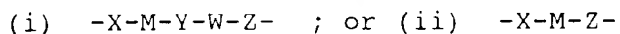
wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; Rⁱ is Ala or (D)2-Nal; R^j is Phe, Gly, Ala or Lys; R^k is Lys or Arg; R^l is (L)Asn or (D)Asn; R^m is Phe, Gly, Ala or Lys, and Y¹ is selected from the group consisting of amide, thioether, thioester and disulfide.

Another aspect of the present invention is a method for the preparation of cyclic peptides of the general Formula (I):



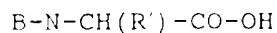
Formula (I)

15 wherein: a-c each independently designates an integer from 1 to 8 or zero; (AA) designates an amino acid residue wherein the amino acid residues in each chain may be the same or different; Q represents H or an acyl group; E represents a hydroxyl group, a carboxyl protecting group or an amino group, or the terminal carboxyl group can be reduced to CH₂-OH; Rⁱ to R^m each designates an amino acid side-chain optionally bound with a specific protecting group; and the lines designate a bridging group of the Formula:



25 wherein M and W are independently selected from the group consisting of amide, thioether, thioester and disulfide; and X, Y and Z are each independently selected from the group consisting of alkylene, substituted alkylene, arylene, homo- or hetero-cycloalkylene and substituted cycloalkylene.

30 This method comprises the steps of incorporating at least one N^α-ω-functionalized derivative of amino acids of Formula (III):



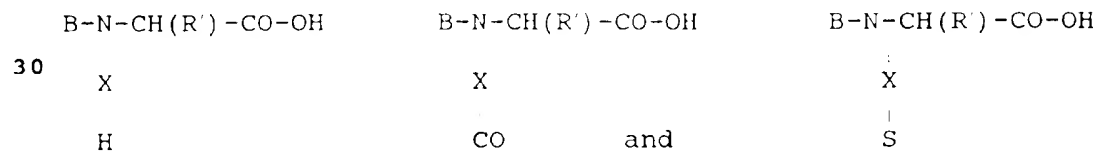
Formula (III)

wherein X is a spacer group selected from the group consisting of alkylene, substituted alkylene, arylene, cycloalkylene and substituted cycloalkylene; Rⁱ is an amino acid side chain, optionally bound with a specific protecting group; B is a protecting group selected from the group consisting of alkyloxy, substituted alkyloxy, or aryl carbonyls; and G is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids and esters, aldehydes, alcohols and alkyl halides; and A is a specific protecting group of G; into a peptide sequence and subsequently selectively cyclizing the functional group with one of the side chains of the amino acids in said peptide sequence or with another ω-functionalized amino acid derivative.

Preferred building units are the co-functionalized amino acid derivatives wherein X is alkylene; G is a thiol group, an amine group or a carboxyl group; R is phenyl, methyl or isobutyl; with the proviso that when G is an amine group, R is other than H.

Further preferred are ω-functionalized amino acid derivatives wherein R is protected with a specific protecting group.

More preferred are ω-functionalized amino acid derivatives of Formula III, wherein G is an amino group, a carboxyl group, or a thiol group:



wherein X, R, A and B are as defined above.

A further preferred embodiment of the invention is a peptide sequence consisting of one or more of the building units defined above.

N^α-ω-functionalized derivative of an amino acid into a peptide sequence and subsequently selectively cyclizing the functional group with one of the side chains of the amino acids in said peptide sequence, or with another ω-functionalized amino acid derivative. Backbone cyclized analogs of the present invention may be used as pharmaceutical compositions and for methods for the treatment of disorders including: post-surgical pain, all types of inflammation, in particular pancreatitis, cancers, endocrine disorders and gastrointestinal disorders.

Therefore, further objects of the present invention are directed to pharmaceutical compositions comprising pharmacologically active backbone cyclized peptide agonists and antagonists prepared according to the methods disclosed herein and a pharmaceutically acceptable carrier or diluent; and methods for the treatment of inflammation, cancer or endocrine disorders and gastrointestinal disorders therewith.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the inhibition of somatostatin (SRIF-14) binding to the different SSTR subtypes, as a function of the concentration of the backbone cyclic somatostatin analog PTR 3046.

Figure 2 is a graph showing the inhibition of somatostatin (SRIF-14) binding to the somatostatin receptors on mouse pituitary AtT20 cell line as a function of the concentration of the backbone cyclic somatostatin analogs PTR 3046 and PTR 3040.

Figure 3 is a graphical comparison of the effects of Octreotide and the backbone cyclic somatostatin analog PTR 3046 on growth hormone release in rats.

Figure 4 is a graphical comparison of the effects of Octreotide and the backbone cyclic somatostatin analog PTR 3046 on insulin release in rats.

5 Figure 5 is a graphical comparison of the effects of Octreotide and the backbone cyclic somatostatin analog PTR 3046 on pancreatic exocrine release following bombesin induction.

10 Figure 6 is a graphical comparison of the antiproliferative effects of PTR 3046 and Octreotide on MiaPaca-2 cells.

15 **DETAILED DESCRIPTION OF THE INVENTION**

The compounds herein described may have asymmetric centers. All chiral, diastereomeric, and racemic forms are included in the present invention. Many geometric isomers of olefins and the like can also be present in the compounds
20 described herein, and all such stable isomers are contemplated in the present invention.

By "stable compound" or "stable structure" is meant herein a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction
25 mixture, and formulation into an efficacious therapeutic agent.

As used herein and in the claims, "alkyl" or "alkylenyl" is intended to include both branched and straight-chain saturated aliphatic hydrocarbon groups having one to ten
30 carbon atoms; "alkenyl" is intended to include hydrocarbon chains of either a straight or branched configuration having two to ten carbon atoms and one or more unsaturated carbon-carbon bonds which may occur in any stable point along the chain, such as ethenyl, propenyl, and the like; and "alkynyl"

which is intended to include both branched and straight-chain saturated aliphatic hydrocarbon groups having one to ten carbon atoms.

may occur in any stable point along the chain, such as ethynyl, propynyl, and the like.

As used herein and in the claims, "aryl" is intended to mean any stable 5- to 7-membered monocyclic or bicyclic or 7-
5 to 14-membered bicyclic or tricyclic carbon ring, any of which may be saturated, partially unsaturated or aromatic, for example, phenyl, naphthyl, indanyl, or tetrahydronaphthyl tetralin, etc.

As used herein and in the claims, "alkyl halide" is
10 intended to include both branched and straight-chain saturated aliphatic hydrocarbon groups having the one to ten carbon atoms, wherein 1 to 3 hydrogen atoms have been replaced by a halogen atom such as Cl, F, Br, and I.

As used herein and in the claims, the phrase
15 "therapeutically effective amount" means that amount of novel backbone cyclized peptide analog or composition comprising same to administer to a host to achieve the desired results for the indications described herein, such as but not limited of inflammation, cancer, endocrine disorders and
20 gastrointestinal disorders.

The term, "substituted" as used herein and in the claims, means that any one or more hydrogen atoms on the designated atom is replaced with a selection from the indicated group, provided that the designated atom's normal
25 valency is not exceeded, and that the substitution results in a stable compound.

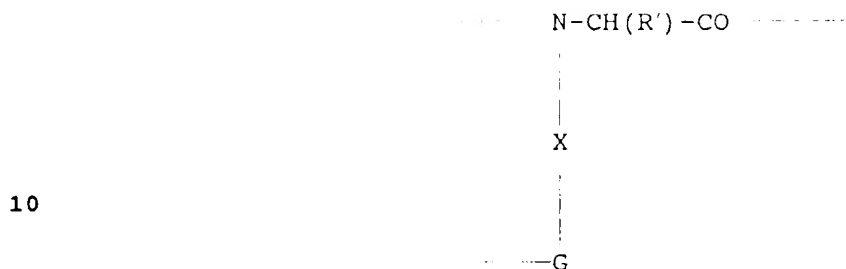
When any variable (for example R, X, Z, etc.) occurs more than one time in any constituent or in any Formula herein, its definition on each occurrence is independent of
30 its definition at every other occurrence. Also, combinations of substituents and/or variables are permissible only if such combinations result in stable compounds.

As used herein "peptide" indicates a sequence of amino acids linked by peptide bonds. The somatostatin peptide

each residue being characterized by having an amino and a carboxy terminus.

A "building unit" indicates an N^ε derivatized α amino acid of the general Formula IV:

5



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Formula (IV)

15 wherein X is a spacer group selected from the group consisting of alkylene, substituted alkylene, arylene, cycloalkylene and substituted cycloalkylene; R' is an amino acid side chain, optionally bound with a specific protecting group; and G is a functional group selected from the group
 20 consisting of amines, thiols, alcohols, carboxylic acids and esters, and alkyl halides; which is incorporated into the peptide sequence and subsequently selectively cyclized via the functional group G with one of the side chains of the amino acids in said peptide sequence or with another ω-
 25 functionalized amino acid derivative.

The methodology for producing the building units is described in international patent application PCT/IB95/00455, which is incorporated in its entirety by way of reference. The building units are abbreviated by the three letter code
 30 of the corresponding modified amino acid followed by the type of reactive group (N for amine, C for carboxyl), and an indication of the number of spacing methylene groups. For example, Gly-C2 describes a modified Gly residue with a carboxyl reactive group and a two carbon methylene spacer,
 35 and Phe-N2 designates a modified Phe residue with an amino reactive group and a two carbon methylene spacer.

As used herein "linear peptide" denotes the peptide sequence that is constructed only of amino acid residues and is devoid of any building units.

As used herein "backbone cyclic peptide" denotes an analog of a linear peptide which contains at least one building unit that has been linked to form a bridge via the alpha nitrogen of the peptide backbone to another building unit, or to another amino acid in the sequence.

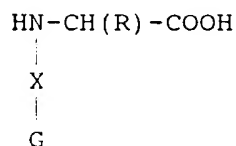
As used herein "pre-cyclic peptide" denotes an analog identical to the cyclic analog except that it is retained in the non-cyclized form to serve as control during the biological or other screening assays. The term non-cyclic can be used interchangeably with the term pre-cyclic. Certain abbreviations are used herein to describe this invention and the manner of making and using it. For instance, AcOH refers to acetic acid, Ada refers to adamantanacetyl, Adac refers to adamantanecarbonyl, Alloc refers to allyloxycarbonyl, Boc refers to the t-butyloxycarbonyl radical, BOP refers to benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate, BSA refers to bovine serum albumin, Cbz refers to the carbobenzyloxy radical, DCC refers to dicyclohexylcarbodiimide, DCM refers to Dichloromethane, Dde refers to 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene-ethyl), DIEA refers to diisopropyl-ethyl amine, DMF refers to dimethyl formamide, DPPA refers to diphenylphosphoryl azide, Dtc refers to 5,5-dimethylthiazolidine-4-carboxylic acid, EDC refers to N-ethyl-N' (dimethylaminopropyl)-carbodiimide, EDT refers to ethanedithiol, Fmoc refers to the fluorenylmethoxycarbonyl radical, GPI refers to guinea pig ileum, HATU refers to [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate, HBTU refers to 1-hydroxybenzotriazolyltetramethyl-uronium hexafluorophosphate, HF refers to hydrofluoric acid, HOBT refers to 1-hydroxybenzotriazole.

the 4-methoxy-2,3,6-trimethylbenzenesulfonyl, NBT refers to nitro blue tetrazolium, NMM refers to N-methylmorpholine, NMP refers to 1-methyl-2-pyrrolidone, PBS refers to phosphate buffered saline, Pmc refers to pentamethylchroman-6-sulfonyl, 5 PNPP refers to p-nitrophenyl phosphate, PPA refers to 1-propanephosphoric acid cyclic anhydride, PyBOP refers to Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, PyBrOP refers to Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate, RT refers to 10 room temperature, SMPS refers to simultaneous multiple peptide synthesis, SRIF refers to Somatotropin Release Inhibitory Factor, TBTU refers to 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, t-Bu refers to the tertiary butyl radical, TFA refers to trifluoroacetic 15 acid, TIS refers to triisopropylsilane, Tpr refers to thiazolidine-4-carboxylic acid, Trt refers to trityl, Ts refers to toluenesulfonyl.

The amino acids used in this invention are those which are available commercially or are available by routine 20 synthetic methods. Certain residues may require special methods for incorporation into the peptide, and either sequential, divergent and convergent synthetic approaches to the peptide sequence are useful in this invention. Natural coded amino acids and their derivatives are represented by 25 three-letter codes according to IUPAC conventions. When there is no indication, the L isomer was used. The D isomers are indicated by "D" before the residue abbreviation. List of Non-coded amino acids: Abu refers to 2-aminobutyric acid, Aib refers to 2-amino-isobutyric acid, Cha refers to 30 cyclohexylalanine, Hcys refer to homocystein, Hyp refers to S-trans-4-hydroxyproline, 1Nal refers to 1-naphthylalanine, 2Nal refers to 2-naphthylalanine, Nva refers to norvaline, Oic refers to octahydroindolecarboxylic acid, Phg refers to phenylglycine, pClPhe refers to p-chloro-phenylalanine, 35 pEPhe refers to p-epi-phenylalanine.

Synthetic Approaches

According to the present invention peptide analogs are cyclized via bridging groups attached to the alpha nitrogens of amino acids that permit novel non-peptidic linkages. In
5 general, the procedures utilized to construct such peptide analogs from their building units rely on the known principles of peptide synthesis; most conveniently, the procedures can be performed according to the known principles of solid phase peptide synthesis. The innovation requires
10 replacement of one or more of the amino acids in a peptide sequence by novel building units of the general Formula:



15

wherein R is the side chain of an amino acid, X is a spacer group and G is the functional end group by means of which cyclization will be effected. The side chain R is the side chain of any natural or synthetic amino acid that is selected
20 to be incorporated into the peptide sequence of choice. X is a spacer group that is selected to provide a greater or lesser degree of flexibility in order to achieve the appropriate conformational constraints of the peptide analog. Such spacer groups include alkylene chains, substituted,
25 branched and unsaturated alkylenes, arylenes, cycloalkylenes, and unsaturated and substituted cycloalkylenes. Furthermore, X and R can be combined to form a heterocyclic structure.

A preferred embodiment of the present invention utilizes alkylene chains containing from two to ten carbon atoms.

30

The terminal (ω) functional groups to be used for cyclization of the peptide analog include but are not limited to:

a. Amines, for reaction with electrophiles such as activated carboxyl groups, aldehydes and ketones (with or

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- b. Alcohols, for reaction with electrophiles such as activated carboxyl groups.
- c. Thiols, for the formation of disulfide bonds and reaction with electrophiles such as activated carboxyl
- 5 groups, and alkyl or substituted alkyl halides.
- d. 1,2 and 1,3 Diols, for the formation of acetals and ketals.
- e. Alkynes or Substituted Alkynes, for reaction with nucleophiles such as amines, thiols or carbanions; free
- 10 radicals; electrophiles such as aldehydes and ketones, and alkyl or substituted alkyl halides; or organometallic complexes.
- f. Carboxylic Acids and Esters, for reaction with nucleophiles (with or without prior activation), such as
- 15 amines, alcohols, and thiols.
- g. Alkyl or Substituted Alkyl Halides or Esters, for reaction with nucleophiles such as amines, alcohols, thiols, and carbanions (from active methylene groups such as acetoacetates or malonates); and formation of free radicals
- 20 for subsequent reaction with alkenes or substituted alkenes, and alkynes or substituted alkynes.
- h. Alkyl or Aryl Aldehydes and Ketones for reaction with nucleophiles such as amines (with or without subsequent reduction), carbanions (from active methylene groups such as
- 25 acetoacetates or malonates), diols (for the formation of acetals and ketals).
- i. Alkenes or Substituted Alkenes, for reaction with nucleophiles such as amines, thiols, carbanions, free radicals, or organometallic complexes.
- 30 j. Active Methylene Groups, such as malonate esters, acetoacetate esters, and others for reaction with electrophiles such as aldehydes and ketones, alkyl or substituted alkyl halides.

It will be appreciated that during synthesis of the

35 peptide these reactions are

Suitable protecting groups for amines are alkyloxy, substituted alkyloxy, and aryloxy carbonyls including, but not limited to, tert butyloxycarbonyl (Boc), Fluorenylmethyloxycarbonyl (Fmoc), Allyloxycarbonyl (Alloc) 5 and Benzyloxycarbonyl (Z).

Carboxylic end groups for cyclizations may be protected as their alkyl or substituted alkyl esters or thio esters or aryl or substituted aryl esters or thio esters. Examples include but are not limited to tertiary butyl ester, allyl 10 ester, benzyl ester, 2-(trimethylsilyl)ethyl ester and 9-methyl fluorenyl.

Thiol groups for cyclizations may be protected as their alkyl or substituted alkyl thio ethers or disulfides or aryl or substituted aryl thio ethers or disulfides. Examples of 15 such groups include but are not limited to tertiary butyl, trityl(triphenylmethyl), benzyl, 2-(trimethylsilyl)ethyl, pixyl(9-phenylxanthen-9-yl), acetamidomethyl, carboxymethyl, 2-thio-4-nitropyridyl.

It will further be appreciated by the artisan that the 20 various reactive moieties will be protected by different protecting groups to allow their selective removal. Thus, a particular amino acid will be coupled to its neighbor in the peptide sequence when the N^α is protected by, for instance, protecting group A. If an amine is to be used as an end 25 group for cyclization in the reaction scheme the N^α will be protected by protecting group B, or an ε amino group of any lysine in the sequence will be protected by protecting group C, and so on.

The coupling of the amino acids to one another is 30 performed as a series of reactions as is known in the art of peptide synthesis. Novel building units of the invention, namely the N^α-ω functionalized amino acid derivatives are incorporated into the peptide sequence to replace one or more of the amino acids. If only one such N^α-ω functionalized 35

instance: (a) an N'-(ω -amino alkylene) amino acid can be linked to the carboxyl group of an aspartic or glutamic acid residue; (b) an N'-(ω -carboxylic alkylene) amino acid can be linked to the ϵ - amino group of a lysine residue; (c) an N'-(ω -thio alkylene) amino acid can be linked to the thiol group of a cysteine residue; and so on. A more preferred embodiment of the invention incorporates two such N'- ω -functionalized amino acid derivatives which may be linked to one another to form N-backbone to N-backbone cyclic peptide analogs. Three or more such building units can be incorporated into a peptide sequence to create bi-cyclic peptide analogs as will be elaborated below. Thus, peptide analogs can be constructed with two or more cyclizations, including N-backbone to N-backbone, as well as backbone to side-chain or any other peptide cyclization.

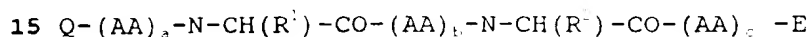
As stated above, the procedures utilized to construct somatostatin analogs of the present invention from novel building units generally rely on the known principles of peptide synthesis. However, it will be appreciated that accommodation of the procedures to the bulkier building units of the present invention may be required. Coupling of the amino acids in solid phase peptide chemistry can be achieved by means of a coupling agent such as but not limited to dicyclohexycarbodiimide (DCC), bis(2-oxo-3oxazolidinyl) phosphinic chloride (BOP-Cl), benzotriazolyl-N-oxytrisdimethyl-aminophosphonium hexafluoro phosphate (BOP), 1-oxo-1-chlorophospholane (Cpt-Cl), hydroxybenzotriazole (HOBT), or mixtures thereof.

It has now been found that coupling of the subsequent amino acid to the bulky building units of the present invention may require the use of additional coupling reagents including, but not limited to: coupling reagents such as PYBOP[®] (Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate), PyBROP[®] (Bromo-tris-pyrrolidino-

(2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate).

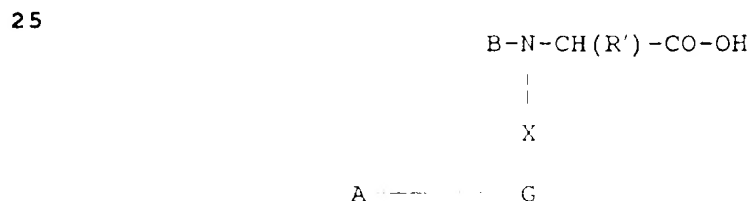
Novel coupling chemistries may be used, such as pre-formed urethane-protected N-carboxy anhydrides (UNCA'S) and pre-formed acyl halides most preferably acyl chlorides. Such coupling may take place at room temperature and also at elevated temperatures, in solvents such as toluene, DCM (dichloromethane), DMF (dimethylformamide), DMA (dimethylacetamide), NMP (N-methyl pyrrolidinone) or mixtures of the above.

One object of the present invention is a method for the preparation of backbone cyclized somatostatin analogs of general Formula (I):



Formula (I)

wherein the substituents are as defined above; comprising the steps of incorporating at least two N^o-ω-functionalized derivatives of amino acids of Formula (III):

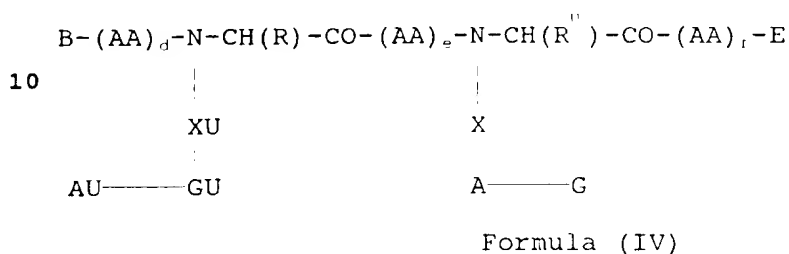


Formula (III)

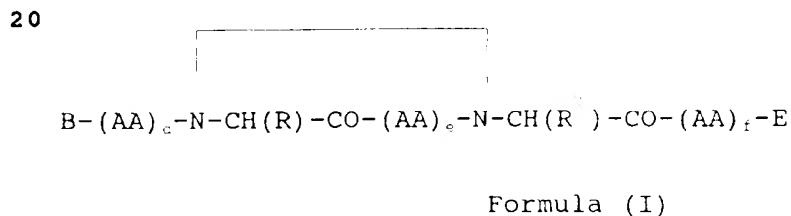
wherein X is a spacer group selected from the group consisting of alkylene, substituted alkylene, arylene, cycloalkylene and substituted cycloalkylene; R is an amino

from the group consisting of alkyloxy, substituted alkyloxy, or aryloxy carbonyls; and G is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids and esters, aldehydes, alcohols and alkyl
 5 halides; and A is a specific protecting group of G;

into an amino acid sequence to yield a compound of Formula:



(ii) selectively removing protecting groups A and A' and reacting the terminal groups G and G' to form a compound of the Formula:



wherein d, e and f are independently an integer from 1 to 10; (AA) is an amino acid residue wherein the amino acid residues in each chain may be the same or different; E is an hydroxyl group, a carboxyl protecting group or an amino
 30 group; R and R' are independently an amino acid side-chain such as H, CH₃, etc.; and the line designates a bridging group of the Formula: -X-M-Y-W-Z-

wherein M and W are independently selected from the
 35 group consisting of disulfide, amide, thioether, imine

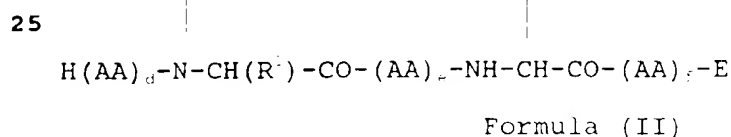
the group consisting of alkylene, substituted alkylene, arylene, cycloalkylene, and substituted cycloalkylene;

(iii) removing all remaining protecting groups to yield a compound of Formula (Ia).

- 5 Bicyclic analogs are prepared in the same manner, that is, by repetition of steps (ii) and (iii). The determination of which residues are cyclized with which other residues is made through the choice of blocking groups. The various blocking groups may be removed selectively, thereby exposing
10 the selected reactive groups for cyclization.

Preferred are methods for the preparation of backbone cyclized peptide analogs of Formula (I) wherein G is an amine, thiol or carboxyl group; R and R' are each other than H, such as CH₃, (CH₃)₂CH-, (CH₃)₂CHCH₂-, CH₃CH₂CH(CH₃)-,
15 CH₃S(CH₂)₂-, HOCH₂-, CH₃CH(OH)-, HSCH₂-, NH₂C(=O)CH₂-, NH₂C(=O)(CH₂)₂-, HOC(=O)CH₂-, HOC(=O)(CH₂)₂-, NH₂(CH₂)₄-, C(NH₂)₂NH(CH₂)₃-, HO-phenyl-CH₂-, benzyl, methylindole, and methylimidazole, and wherein E is covalently bound to an insoluble polymeric support.

- 20 Another object of the present invention is a method for the preparation of backbone cyclized peptide analogs of Formula (II):



- wherein the substituents are as defined above;
30 comprising the steps of: incorporating at least one ω-functionalized amino acid derivative of the general Formula (III):



A G

Formula (III)

5 wherein X is a spacer group selected from the group consisting of alkylene, substituted alkylene, arylene, cycloalkylene and substituted cycloalkylene; R is the side chain of an amino acid, such as H, CH₃, etc.; B is a
 10 protecting group selected from the group consisting of alkyloxy, substituted alkyloxy, or aryloxy carbonyls; and G is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids and esters or
 15 alkyl halides; and A is a protecting group thereof; into a peptide sequence and subsequently selectively cyclizing the functional group with one of the side chains of the amino acids in said peptide sequence.

 Preferred is the method for the preparation of backbone cyclized peptide analogs of Formula (II) wherein G is a
 20 carboxyl group or a thiol group; R is CH₃, (CH₃)₂CH-, (CH₃)₂CHCH₂-, CH₃CH₂CH(CH₃)-, CH₃S(CH₂)₃-, HOCH₂-, CH₃CH(OH)-, HSCH₂-, NH₂C(=O)CH₂-, NH₂C(=O)(CH₂)₂-, HOC(=O)CH₂-, HOC(=O)(CH₂)₂-, NH₂(CH₂)₄-, C(NH₂)₂NH(CH₂)₃-, HO-phenyl-CH₂-, benzyl, methylindole, and methylimidazole, and wherein E is
 25 covalently bound to an insoluble polymeric support.

Preparation of Peptides with Backbone to Side Chain Cyclization.

 One preferred procedure for preparing the desired
 30 backbone cyclic peptides involves the stepwise synthesis of the linear peptides on a solid support and the backbone cyclization of the peptide either on the solid support or after removal from the support. The C-terminal amino acid is bound covalently to an insoluble polymeric support by a carboxylic acid ester or other linkages such as amides.

such chemistries as Fmoc and Boc and include for example PAM resin, HMP resin and chloromethylated resin. The resin bound amino acid is deprotected for example with TFA and to it is coupled the second amino acid, protected on the N^α for example by Fmoc, using a coupling reagent like BOP. The second amino acid is deprotected using for example piperidine 20% in DMF. The subsequent protected amino acids can then be coupled and deprotected at ambient temperature. After several cycles of coupling and deprotection that gives peptide, an amino acid having for example carboxy side chain is coupled to the desired peptide. One such amino acid is Fmoc-aspartic acid t-butyl ester. After deprotection of the N^α Fmoc protecting group, the peptide is again elongated by methods well known in the art. After deprotection a building unit for backbone cyclization is coupled to the peptide resin using for example the coupling reagent BOP. One such building unit is for example Fmoc-N^α-(ω-Boc-amino alkylene)amino acid. After deprotection the peptide can then be elongated, to the desired length using methods well known in the art. The coupling of the protected amino acid subsequent to the building unit is performed by such coupling agents exemplified by PyBROP to ensure high yield. After the linear, resin bound peptide, has been prepared the co-alkylene-protecting groups, for example Boc and t-Bu, are removed by mild acid such as TFA. The resin bound peptide is then divided into several parts. One part is subjected to on-resin cyclization using for example TBTU as cyclization agent in DMF to ensure high yield of cyclization, to give the N-backbone to side chain cyclic peptide resin. After cyclization on the resin the terminal amino protecting group is removed by agents such as piperidine and the backbone to side chain cyclic peptide is obtained after treatment with strong acid such as HF. Alternatively, prior to the removal of the backbone cyclic peptide from the resin, the terminal

25

adamantyl carboxylic acid activated by coupling agents such as BOP.

The other part of the peptide-resin undergoes protecting of the side chains used for cyclization, for example the ω -
5 amino and carboxy groups. This is done by reacting the ω -
amino group with for example Ac_2O and DMAP in DMF and
activating the free ω -carboxy group by, for example, DIC and
HOBT to give the active ester which is then reacted with, for
example, CH_3NH_2 to give the non-cyclic analog of the cyclic
10 peptide. Removal of the peptide from the resin and
subsequent removal of the side chains protecting groups by
strong acid such as HF to gives the non-cyclic analog of the
backbone to side chain cyclic peptide.

The linear and/or non-cyclic analogs are used as
15 reference compounds for the biological activity of their
corresponding cyclic compounds.

Synthetic Approach for Generation of Backbone Cyclized
Somatostatin Libraries.

20 The general methodology for preparing the cyclic peptide
libraries of this invention involves solid phase peptide
synthesis using an orthogonal protection scheme which allows
for chain elongation, selective removal of the protecting
groups, cyclization of the protected peptides and removal of
25 all side-chains protecting groups with or without cleavage
from the resin. It is desirable that the various peptide
sequences be present in the libraries in substantially equal
amount.

The coupling reactions are performed by methods to
30 create amide or ester bonds and are performed by methods
familiar in the art as described herein. Typical coupling
reagents are carbodiimides, activated anhydrides and esters
and acyl halides. Reagents such as EDC, DCC, DPPA, PPA, BOP,
PyBOP, PyBrop, HATU, HBTU, TBTU, HOBT and
35

After completion of the solid phase peptide elongation, by any scheme, portions of the peptide are cyclized via the bridging groups attached to the backbone amine bond nitrogens of the building units. It is preferable that a portion is
5 retained in the non-cyclized form to serve as control during the biological or other screening assays. This portion of the peptide analog library, which contains the building units identical to those of the backbone cyclized library, but is devoid of the conformational constraint of the latter, is
10 referred to as the "pre-cyclic". Alternatively, in any of the synthesis schemes, the backbone cyclization step may be performed and additional coupling cycles of amino acid residues may then be carried out.

Portions of the peptide may be cleaved from the resin
15 and protecting groups removed, as required prior to assay of biological activity. The peptides are cleaved from the resin support by methods known in the art, the precise method being dependent upon the characteristics of the resin. It will be understood by those skilled in the art that the removal of
20 certain protecting groups may occur simultaneously with cleavage of the peptide from the resin.

Typically the coupling between the resin and the first amino acid will form an ester bond, which will yield a carboxylic acid group on the peptide when it is cleaved from
25 the resin. HMPB, Rink, PAM, Hycram and hydroxymethyl resins are exemplary. In addition, the carboxy terminal amino acid group may be converted to an amide, an ester or reduced to a terminal alcohol.

The reactive functional groups of the side chains of
30 each amino acid or peptide are suitably protected as known in the peptide art. For example, the Boc, Cbz or Fmoc group may be used for protection of an amino group, especially an α -amino group. An alkyl (e.g., t-Bu, Me), cHex, benzyl or allyl ester may be used for the protection of the side chain

25

- residues; or the hydroxyl of Tyr, Ser or Thr. Cys and other sulfur-containing amino acids may also be protected by the Acn group or by formation of a disulfide with a thioalkyl (e.g., ethyl mercaptan) or thioaryl group. The
- 5 benzyl/benzyloxymethyl, or a suitably substituted benzyl/benzyloxymethyl, Boc or formyl group may be used for protection of the imidazolyl group of His; and the Pmc, nitro, or a suitably substituted benzene-sulfonyl group (e.g., Ts, Mts) for protection of the guanidino nitrogen or
- 10 Arg. The phthalamido, Boc, Fmoc, Alloc carbobenzyloxy or benzyl group, or suitably substituted benzyl or benzyloxy group, may be used for protecting the ϵ -amino group of lysine. Suitable substitution of the carbobenzyloxy or benzyl protecting groups is substitution with one to five
- 15 chloro, bromo, nitro, methoxy or methyl groups, usually ortho and/or para, and is used to modify the reactivity of the protective group. These protective groups are removed by such methods as catalytic hydrogenation, sodium in liquid ammonia, hydrazine, base, TFA or HF treatment, as known in
- 20 the art. The choice of side chain protecting groups is chosen so that they will not be removed under conditions which are used to deprotect the reactive functional group used in the coupling reaction (e.g., generally the α -amino group) to form the peptide backbone of the peptide chain.
- 25 The protective group of the reactive functional group is removed prior to coupling each successive amino acid.

The bridging groups of the building units (i.e., G in Formula IV) are used according to the present invention with an orthogonal protection scheme, such that these protecting

30 groups can be removed selectively, under conditions which do not affect the protecting groups on the side chains or cleavage of the peptide from the resin. This enables backbone cyclization on the resin, which is preferred synthetically. Alternatively, the fully protected peptide

solution after selective removal of the protecting groups of the building units.

The cyclization reaction is carried out by means of selectively coupling the bridging group of one building unit
5 to a bridging group of another building unit or amino acid side chain. By way of example, PyBOP is a particularly useful reagent for conducting the coupling reaction, in case of formation of an amide bond. To form a disulfide bridge oxidative conditions are used.

10 In a most preferred embodiment according to the present invention, the amino acid sequence scaffold is based on known active sequences from natural or synthetic peptides having somatostatin activity. It will thus be possible to further improve the activity of such known sequences upon
15 rigidification of the active conformer.

Amino acids in certain positions are replaced by Backbone-Cyclization Building-Units or by natural and non-natural trifunctional amino acids such as Asp, Glu, Cys, Hcys, Lys, Orn and their D counterparts. Thus positional as
20 well as structural scans are performed by changing the position of cyclization, the link of the ring to the backbone, the chirality at the position of cyclization, the ring forming bond, the ring size and the exact placement of the bond within the ring. These variations may also be
25 performed in conjunction with changing the amino acid sequence of the peptide.

General synthesis of libraries of somatostatin analogs.

To determine the optimum compounds, a library of
30 differently constrained analogs is generated and then screened. The libraries were synthesized on Tentagel amide Resin (substitution level of 0.2-0.3 mmol/g) using conventional solid-phase peptide synthesis (known to those skilled in the art). In most cases NMP was used as a

solvent and the reaction was carried out at room temperature.

In each coupling step where more than one amino acid had to be coupled, the resin was divided into the appropriate number of portions and different amino acid was added to each portion. Coupling was performed, twice for each position
5 with 3 molar excess of each amino acid, 3 molar excess of PyBrop and 6 molar excess of DIEA for duration of 1-16 hours. All amino acids were protected with FMOC in their α -amine. Side-chain protections were as follow: His(Trt); Lys(Boc or Dde); Orn(Boc); Ser(tBu); Thr(tBu); Tyr(tBu).

10 After double coupling, the resin portions were washed, recombined and FMOC deprotection was performed using 20% piperidine in NMP for total of 20-40 minutes. After additional washes the resin was divided again (if necessary) for the coupling of the next amino acid/s.

15 Before cyclization, the Allyl/Alloc protection of the amine and carboxyl of the building units were removed by treatment with a solution of 2 mole equivalents (one for each Allyl/Alloc molecule in peptide), of $\text{Pd}(\text{PPh}_3)_2$ dissolved in chloroform containing 2.5% AcOH and 5% NMM for 2-2.5 hours or
20 twice for 1 hour, resins were washed with the above solvent without the palladium before and after treatment, additional washes with NMP were made at the end of the removal process.

The peptides were cleaved from the resin portions after washes with DCM, by double treatment with TFA 70%, H_2O 5%, TIS
25 1%, EDT 2.5%, DCM (mixture A) or TFA 70%, H_2O 5%, TIS 1%, Phenol 5%, DCM (mixture B) or 60% TFA, 10% H_2O and 30% DCM (mixture C) plus additional wash with neat TFA. The three cleavage solutions of each resin portion were collected together, evaporated with nitrogen stream, 0.5-1 ml of H_2O
30 were added to each sample that was then freeze-dried. The peptide mixtures were then partially purified on C-18 SEP-PAK (Millipore Corp.) using 0.1% acetic acid or TFA in H_2O as buffer A and 50-80% CH_3CN in 0.1% acetic acid / H_2O as buffer B and freeze-dried.

35

The building units are abbreviated by the three letter code of the corresponding modified amino acid followed by the type of reactive group (N for amine, C for carboxyl), and an indication of the number of spacing methylene groups. For example, Gly-C2 describes a modified Gly residue with a carboxy reactive group and a two carbon methylene spacer, and Phe-N3 designates a modified phenylalanine group with an amino reactive group and three carbon methylene spacer.

10 General screening of somatostatin analogs

Somatostatin analogs synthesized are typically tested in vitro for their inhibition of the natural peptide (SRIF-14) binding to its 7-transmembranal receptors, and for their influence on second messengers and cell growth; and in vivo for inhibition of hormones and enzyme secretion.

The analogs are further tested in vitro for their influence on cyclic adenosine monophosphate (cAMP) levels, tyrosine phosphatase activity, growth hormone secretion, and cell growth. The libraries are further tested in vivo for the inhibition of growth-hormone release, and amylase, gastric acid, insulin and glucagon secretion in animals.

Metabolic stability tests as parameter for selection:

Analogues are tested for stability by their resistance to enzymatic degradation by incubation in serum or in tissue homogenate, separation of the proteins and recording of the peptide peaks by HPLC before and after incubation. The peptide peaks that are not changed with increased incubation time are most stable. These peaks are separated and characterized by mass spectrometry, N-terminal sequence and comparison to purified peptide peaks. In this way the most stable peptides from library or sub-library are rapidly identified.

Conformationally constrained somatostatin analogs

The following examples are intended to illustrate how to make and use the compounds and methods of this invention and are in no way to be construed as a limitation.

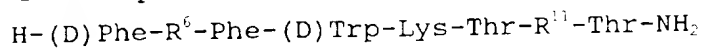
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EXAMPLESsynthetic examples

Different series of somatostatin analogs were synthesized, either as individual backbone cyclized peptides or as libraries.

Three series of octapeptide somatostatin analogs corresponding to general Formula (Va) of the present invention were individually synthesized, characterized, and tested for biological activity.

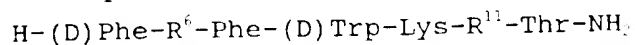
- 15 1) The first series of compounds corresponds to the general Formula (Va), wherein R^5 is (D)Phe; R^7 is Phe; R^{10} is Thr; and R^{11} is Thr. This series, therefore, comprises compounds of the specific formula:



- 20 wherein R^6 and R^{11} are N^ω -functionalized alkylene amino acid building units.

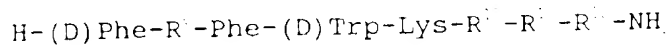
- 2) The second series of compounds corresponds to the general Formula (Va), wherein R^5 is (D)Phe; R^7 is Phe; R^{10} is absent; and R^{11} is Thr. This series, therefore, comprises

25 compounds of the specific formula



wherein R^6 and R^{11} are N^ω -functionalized alkylene amino acid building units.

- 3) The third series of compounds corresponds to the
- 30 general Formula (Va), wherein R^5 is (D)Phe; and R^7 is Phe. This series, therefore, comprises compounds of the specific formula



wherein R^6 and R^{11} are N^ω -functionalized alkylene amino acid

The structures of these novel synthetic peptide analogs into which N^α-functionalized amino acid building units were incorporated, are summarized in Tables 1, 2 and 3. In these three series, the building units used were glycine building
5 units in which the bridging groups, attached via the alpha nitrogens to the peptide backbone, were varied.

For the sake of simplicity, these three series are referred to herein as the SST Gly⁶, Gly¹¹; SST Gly⁶, Gly¹⁰; and SST Gly⁶ Gly¹¹ R¹⁰ R¹¹, respectively.

10 In each series, the position of the cyclization points was constant; in the first and second series the length and direction of the bridge was varied, whereas in the third series the bridge was constant and residues at positions 10 and 12 were varied. Thus, C2, N2 refers to a bridge
15 consisting of an amide bond in which the carbonyl group is closer to the amino end of the peptide and which contains a two carbon methylene group between the bridge amide and each of the backbone nitrogens involved in the bridge.

Peptide assembly was carried out either manually or with
20 an automatic peptide synthesizer (Applied Biosystems Model 433A). Following peptide assembly, de-protection of bridging groups that form the cyclization arms was carried out with Pd(PPh₃)₄ (palladium tetrakis triphenyl phosphine) in the case of Allyl/Alloc protecting groups or with TFA in the case of
25 tBu/Boc protecting groups. For obtaining the non-cyclic analog, the peptides were cleaved from the resin at this stage. Cyclization of the peptides was carried out with PyBOP. Cleavage of the peptides from the polymeric support was carried out with suitable reagents depending on the type
30 of resin used, e.g., with TFA for Rink amide type resins and with HF for mBHA (para-methyl benzhydryl amine) type resins. The crude products were characterized by analytical HPLC. The peptides were purified by preparative reversed phase HPLC. The purified products were characterized by analytical

Table 1. SST Gly⁶ Gly¹¹ analogs

Example No.	Bridging Groups	Compound Number	Method	Crude Yield
1	C1,N2 Cyclic	DE-3-32-4	1	NA**
2	C1,N2 Non-cyclic	DE-3-32-2	1	NA
3	C1,N3 Cyclic	PTR 3004	2	79 mg
4	C1,N3 Non-cyclic	PTR 3005	2	34 mg
5	C2,N2 Cyclic	PTR 3002	1	NA
6	C2,N2 Non-cyclic	PTR 3001	1	NA
7	C2,N3 Cyclic	PTR 3007	2	40 mg
8	C2,N3 Non-cyclic	PTR 3008	2	40 mg
9	N2,C2 Cyclic	YD-9-166-1	2	NA
10	N2,C2 Non-cyclic	YD-9-168-1	2	NA
11	N3,C2 Cyclic	PTR 3010	2	100 mg
12	N3,C2 Non-cyclic	PTR 3011	2	NA
13	Linear*	PTR 3003	3	96 mg

* Linear refers to the identical sequence with underivatized Gly residues in place of R⁶ and R¹¹.
 ** NA denotes not available.

Table 1 methods:

- 1) Manual synthesis on mBHA resin. HF cleavage.
- 2) Manual synthesis on Rapp TentaGel resin. TFA cleavage.
- 3) Rink amide resin; assembly in automated peptide synthesizer, 0.1 mmol scale.

Table 2 SST Gly⁶, Gly¹⁰ analogs

Example No.	Bridging Groups	Compound Number	Method	Crude Yield
14	C1,N2 Cyclic	YD-9-171-3	1	20 mg
15	C1,N2 Non-cyclic	YD-9-171-2	1	10 mg
16	C1,N3 Cyclic	YD-9-175-3	1	44.9 mg
17	C1,N3 Non-cyclic	YD-9-175-2	1	25.4 mg
18	C2,N2 Cyclic	PTR 3019	1	40 mg
19	C2,N2 Non-cyclic	PTR 3020	1	26 mg
20	C2,N3 Cyclic	YD-5-28-3	3	101.5 mg
21	C2,N3 Non-cyclic	YD-5-28-2	3	48.3 mg
22	N2,C2 Cyclic	PTR 3016	2	60 mg
23	N2,C2 Non-cyclic	PTR 3017	2	40 mg

26	* Linear	PTR 3021	1	100 mg
27	N3,C2 Cyclic**	PTR 3013		67 mg
28	N3,C2 Non-cyclic**	PTR 3014		48 mg

- 5 * Linear refers to the identical sequence with Gly residues in place of R⁶ and R¹².
- ** These analogs comprise of the same SST sequence in which the N terminal D-Phe¹ is absent and the N-terminus is acetylated.

Table 2 methods:

- 10 1) Assembly in automated peptide synthesizer, 0.1 mmol scale. (HBTU).
- 2) Manual synthesis; PyBrop.
- 3) Assembly in automated peptide synthesizer, 0.25 mmol scale. (HBTU).

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Table 3. Somatostatin analogs based on: H-(D)Phe-R'-Phe-(D)Trp-Lys-R¹⁰-R¹¹-R¹²-NH₂

	Example No.	Analog	R ¹⁰	R ¹²	Yield (mg)
5	29	GGP-22-65	Nva	Thr	300
	30	GGP-22-63	Val	Thr	300
	31	GGP-22-61	Abu	Thr	300
	32	GGP-22-59	Ser	Thr	350
10	33	GGP-22-75	Thr	Nal	125
	34	GGP-22-81	Val	Nal	210
	35	GGP-22-82	Abu	Nal	200
	36	GGP-22-77	Ser	Nal	190
	37	GGP-22-83	Thr	(D)Nal	68
	38	GGP-22-89	Val	(D)Nal	58
15	39	GGP-22-87	Abu	(D)Nal	65
	40	GGP-22-85	Ser	(D)Nal	58

Example 41. Detailed synthesis of SST Gly⁶, Gly¹⁰ N₃,C₂ analog.

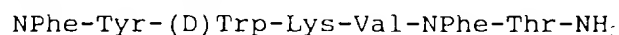
Five grams of Rink amide resin (NOVA) (0.49 mmol/g),
 were swelled in N-methylpyrrolidone (NMP) in a reaction
 vessel equipped with a sintered glass bottom and placed on a
 shaker. The Fmoc protecting group was removed from the resin
 by reaction with 20% piperidine in NMP (2 times 10 minutes,
 25 ml each). Fmoc removal was monitored by ultraviolet
 absorption measurement at 290 nm. A coupling cycle was
 carried out with Fmoc-Thr(OtBu)-OH (3 equivalents) PyBrop (3
 equivalents) DIEA (6 equivalents) in NMP (20 ml) for 2 hours
 at room temperature. Reaction completion was monitored by
 the qualitative ninhydrin test (Kaiser test). Following
 coupling, the peptide-resin was washed with NMP (7 times with
 25 ml NMP, 2 minutes each). Capping was carried out by
 reaction of the peptide-resin with acetic anhydride (capping
 mixture: HOBt 400 mg, NMP 20 ml, acetic anhydride 10 ml,
 DIEA 4.4 ml) for 0.5 hours at room temperature. After

- Phe-OH was coupled in the same manner, and the Fmoc group removed, as above. The peptide resin was reacted with Fmoc-Gly-C2 (Allyl) building unit: coupling conditions were as above. Fmoc removal was carried out as above. Fmoc-
- 5 Lys(Boc)-OH was coupled to the peptide resin by reaction with HATU (3 equivalents) and DIEA (6 equivalents) at room temperature overnight and then at 50°C for one hour. Additional DIEA was added during reaction to maintain a basic medium (as determined by pH paper to be about 9). This
- 10 coupling was repeated. Coupling completion was monitored by the Fmoc test (a sample of the peptide resin was taken and weighed, the Fmoc was removed as above, and the ultraviolet absorption was measured). Fmoc-D-Trp-OH was coupled to the peptide resin with PyBrop, as described above. Following
- 15 Fmoc removal, Fmoc-Phe-OH was coupled in the same way. Synthesis was continued with one-fifth of the peptide resin. Following Fmoc removal, the second building unit was introduced: Fmoc-Gly-N3(Alloc)-OH by reaction with PyBrop, as described above. Capping was carried out as described above.
- 20 Following Fmoc removal, the peptide-resin was divided into two equal portions. Synthesis was continued with one of these portions. Boc-D-Phe-OH was coupled by reaction with HATU, as described above for Fmoc-Lys(Boc)-OH. Capping was carried out as above.
- 25 The Allyl and Alloc protecting groups were removed by reaction with $\text{Pd}(\text{PPh}_3)_4$ and acetic acid 5%, morpholine 2.5% in chloroform, under argon, for 2 hours at room temperature. The peptide resin was washed with NMP as above. Two-thirds of the resin were taken for cyclization. Cyclization was
- 30 carried out with PyBOP 3 equivalents, DIEA 6 equivalents, in NMP, at room temperature overnight. The peptide resin was washed and dried. The peptide was cleaved from the resin by reaction with TFA 81.5%, phenol 5%, water 5%, EDT 2.5%, TIS (tri-isopropyl-silane) 1%, and 5% methylene chloride, at 0°C
- 35 for 15 minutes.

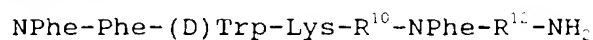
resin was washed with a small volume of TFA. The filtrate was placed in a rotary evaporator and all the volatile components were removed. An oily product was obtained. It was triturated with ether and the ether decanted, three 5 times. A white powder was obtained. This crude product was dried. The weight of the crude product was 93 mg.

Additional series of novel backbone cyclized somatostatin analogs according to Formula Vb were synthesized individually 10 including the following:

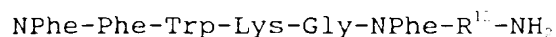
1) heptapeptide series:



15 2) heptapeptide series:



3) Heptapeptides series:



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In the first series (table 4), the length and direction of the bridge was varied, whereas in the second (table 5), and third (table 6), series the residues at positions 10 and/or 12 were varied.

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Table 4. Heptapeptide somatostatin analogs based on:
 R^6 -Tyr-(D)Trp-Lys-Val- R^{11} -Thr-NH₂

Example No.	Analog	R^6	R^{11}	Yield (mg)
42*	GGP-22-151	Phe-N2	Phe-C3	290
43	GGP-22-135	Phe-N3	Phe-C3	25
44	GGP-22-159a	Phe-N2	Phe-C2	28
45	GGP-22-159b	Phe-N3	Phe-C2	30
46	GGP-22-161a	Phe-C2	Phe-N2	56
47	GGP-22-161b	Phe-C3	Phe-N2	65
48	GGP-22-163a	Phe-C1	Phe-N3	61
49	GGP-22-163b	Phe-C1	Phe-N3	68
50	GGP-22-163c	Phe-C3	Phe-N3	10

*PTR-3046

Table 5. Additional heptapeptide somatostatin analogs based on: R^6 -Phe-(D)Trp-Lys- R^{10} - R^{11} - R^{12} -NH₂

Example No.	Analog	R^6	R^{10}	R^{11}	R^{12}	Yield (mg)
51	GGP-22-53	Phe-N2	Val	Phe-C3	Val	200
52	GGP-22-55	Phe-N2	Val	Phe-C3	Thr	460
53	GGP-22-41	Phe-N2	Thr	Phe-C3	Thr	120
54	GGP-22-137	Phe-N3	Thr	Phe-C3	Thr	30
55	GGP-22-37	Phe-N2	Thr	Phe-C3	Val	146

Table 6. Heptapeptide somatostatin analogs based on: R^6 -Phe-Trp-Lys-Gly- R^{10} - R^{11} -NH₂

Example No.	R^6	7	R^7	R^8	R^{10}	R^{11}	R^{12}
56	Phe-N2	Phe	Trp	Lys	Gly	Phe-C3	Thr
57	Phe-N2	Phe	Trp	Lys	Gly	Phe-C3	Val
58	Phe-N2	Phe	Trp	Lys	Gly	Phe-C3	Ala
59	Phe-N2	Phe	Trp	Lys	Gly	Phe-C3	b-Ala

Example 61. Detailed synthesis of PTR 3046

1g Rink Amide MBHA resin (NOVA) (0.55 mmol/g), were swelled for 1.5h in NMP in a reaction vessel equipped with a sintered glass bottom and placed on a shaker. The Fmoc protecting group was removed from the resin by reaction with 20% Piperidine in NMP (2 times 15 minutes, 5 ml each). Fmoc removal was monitored by Ninhydrin test. A coupling cycle was carried out with Fmoc-Thr(OtBu)-OH (4 equivalents) PyBrop (4 equivalents) DIEA (12 equivalents) in NMP (5 ml) for 0.5 hour at room temperature. Reaction completion was monitored by the qualitative Ninhydrin test (Kaiser test). Following coupling the peptide-resin was washed with NMP (3 times with 5 ml NMP, 5 ml DCM and 5 ml NMP for 2 minutes). Capping was carried out by reaction of the peptide-resin with acetic anhydride (capping mixture: HOAt 40 mg, NMP 5 ml, acetic anhydride 1 ml, DIEA 0.5 ml and DMAP(cat)) for 0.5 hours at room temperature. After capping NMP washes were carried out as above). Fmoc removal was carried out as above. Fmoc-Phe(C3)-Allyl BU was coupled (BU 2eq., PyBrop 2 eq. DIEA 6 eq., NMP 5 ml, 0.5 h. Fmoc removal was carried out as above. The peptide resin was washed as above. The peptide resin was reacted with Fmoc-Val-Cl (4 eq., Colidine 12 eq., 1h, 38C) by double coupling. Coupling completion was monitored due to conversion of dipeptide to tripeptide (a sample of the peptide resin was cleaved and crude tripeptide was injected into HPLC (0.1% water/acetonitrile). Fmoc removal was carried out as above. Fmoc-Lys(Boc)-OH was coupled to the peptide resin by reaction conditions as for Fmoc-Thr(OtBu)-OH (see above). Coupling completion was monitored by Ninhydrin test. Fmoc-D-Trp-OH was coupled to the peptide resin with PyBrop, as described above. Following Fmoc removal, Fmoc-Tyr(tBu)-OH was coupled in the same way. Following Fmoc removal, the second building unit was introduced: Fmoc-Phe-N₂(Alloc)-OH by reaction with PyBrop, as described for Fmoc-

hours at room temperature. The peptide resin was washed as above. Cyclization was carried out with PyBOP, 3 equivalents, DIEA 6 equivalents, in NMP, at room temperature for 0.5h. The peptide resin was washed as above. Following
 5 Fmoc removal the peptide resin was washed (DCM 3 x 5 ml), dried and cleaved from the resin by reaction with TFA 94%, water 2.5%, EDT 2.5%, TIS (tri-isopropyl-silane) 1%, at 0°C for 15 minutes and 1.5 hours at room temperature. The mixture was filtered and the resin was washed with a small
 10 volume of TFA. The filtrate was placed in a rotary evaporator and all the volatile components were removed. An oily product was obtained. It was triturated with ether and the ether decanted. Yellow powder was obtained. This crude product was dried. The weight of the crude product was 290
 15 mg.

Additional examples of individual somatostatin analogs

Additional individual examples of novel somatostatin analogs
 20 produced according to the invention are summarized in Table 7.

Table 7. Additional somatostatin analogs synthesized.

Example No.	R ⁵	R ⁶	7	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²
25 61		Phe-N2	Tyr	(D)Trp	Lys	Val	Phe-C3	
62		Phe-N2	Tyr	(D)Trp	Lys	Val	Phe-C3	
63		Phe-N2	pNO.Phe	(D)Trp	Lys	Val	Phe-C3	Thr
30 64		Phe-N2	Tyr	(D)Trp	Lys	(D)Val	Phe-C3	Thr
65		Phe-N2	pClPhe	(D)Trp	Lys	Val	Phe-C3	Thr
66		Phe-N2	Nal	(D)Trp	Lys	Val	Phe-C3	Thr
67	(D)Phe	Phe-N2	Tyr	(D)Trp	Lys	(D)Val	Phe-C3	

Libraries of Somatostatin analogs

Position numbers of amino acids in the somatostatin sequence are based on the native somatostatin peptide (SRIF-14, Raynor et. al. ibid).

5

Example 68: VH-SST1 library

The library was designed to contain 40 backbone cyclic peptides in five final sub-libraries, each containing 8 different peptides.

- 10 Before the last coupling step (R^7), the resin was split into 5 portions, coupling was performed for each portion with different AA and left as separate sub-libraries for the following steps. These five portions are the sub-libraries as described in the table 8 which indicates all the residues
15 used at each position for generation of the 40 analogs.

Table 8. The composition of VH-SST1 library

20 Sub-library	Residues per position							
	R^5	R^6	R^7	R^8	R^9	R^{10}	R^{11}	R^{12}
A	DPhe	Gly-C2	Phe	DTrp	Lys	Gly	Gly-N2	Val
B	Pro			Leu	Pro	Val		
C	Val							
D	Leu							
E	Gly							

25 Example 69: IG-SST1 library

- In this library, the bridge was constant between position 6 with Gly-C2 and position 10 with Gly-N2. The library contains 36 peptides in 4 sub-libraries, which differ in their R^7 residue. The composition of this library is shown
30 in table 9.

Table 9. The composition of IG-SST1 library

Sub-library	Residues per position							
	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²
A	DPhe	Gly-C2	Phe	DTrp	Lys	Gly-N2	Phe	Val
B	Phe			Phe			Ala	
C	Dtrp			Leu			Leu	
D	Trp							

Example 70: YS-SST1 library

This library represents 16 analogs in 4 sub-libraries as described in table 10. The sub-libraries are defined by four different bridge groups (between position R⁶ and position R¹¹ or R¹⁰), and each sub-library contains four analogs.

Table 10. The composition of YS-SST1 library

Sub-library	Residues per position							
	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²
A1-2	DPhe	Gly-C1 Gly-C2	Phe	DTrp	Lys	Thr	Gly-N2 Gly-N3	Thr
A3-4	DPhe	Gly-N2 Gly-N3	Phe	DTrp	Lys	Thr	Gly-C1 Gly-C2	Thr
B1-2	DPhe	Gly-C2 Gly-C2	Phe	DTrp	Lys	Gly-N2 Gly-N3	Phe	Thr
B3-4	DPhe	Gly-N1 Gly-N3	Phe	DTrp	Lys	Gly-C1 Gly-C2	Phe	Thr

Example 71: YS-SST2 library

The library contains 48 peptides in 4 sub-libraries. The sub-libraries differ in their R⁷ residue and contain 12 peptides each. The composition of this library is shown in table 11.

Table 11. The composition of YS-SST2 library.

Sub-library	Residues per position							
	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²
A	DPhe	Gly-N3	Phe	DTrp	Lys	Thr	Gly-C2	Thr
B			Tyr			Ser		2NaI
C			pClPhe			Val		p2NaI

Example 72: YS-SST3 library

The library contains 12 peptides in 2 sub-libraries. The sub-libraries differ in their R⁵ building unit and contain 6 peptides each. The composition of this library is shown in table 12.

Table 12: The composition of YS-SST3 library

Sub-library	Residues per position							
	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²
A	DPhe	Phe-N2	Phe	DTrp	Lys	Thr	Phe-C2	Thr
B	Gly	Phe-N3				Ser		
						Gly		

Example 73: YS-SST4 library

This library contains 48 peptides in two sub-libraries. Sub-library B differs from A by the existence of Thr at R⁵ position as described in Table 13.

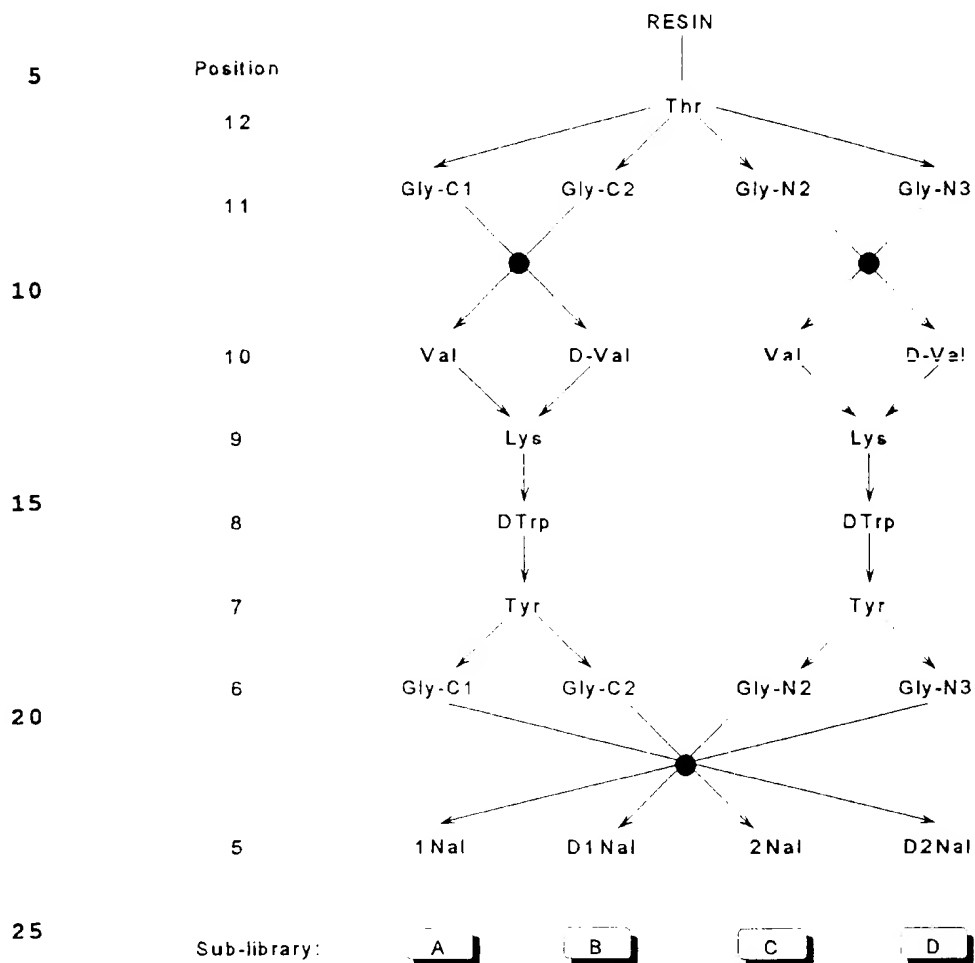
Table 13. The composition of YS-SST4 library

Sub-library	Residues per position							
	R ¹²	R ¹¹	R ¹⁰	R ⁹	R ⁸	R ⁷	R ⁶	R ⁵
A		Phe-C2	DVal DAla DLeu	DLys DOrn	Trp Thi	DTyr	Gly-N2 Gly-N3	Phe
B	Thr	Phe-C2	DVal DAla DLeu	DLys DOrn	Trp Thi	DTyr	Gly-N2 Gly-N3	Phe

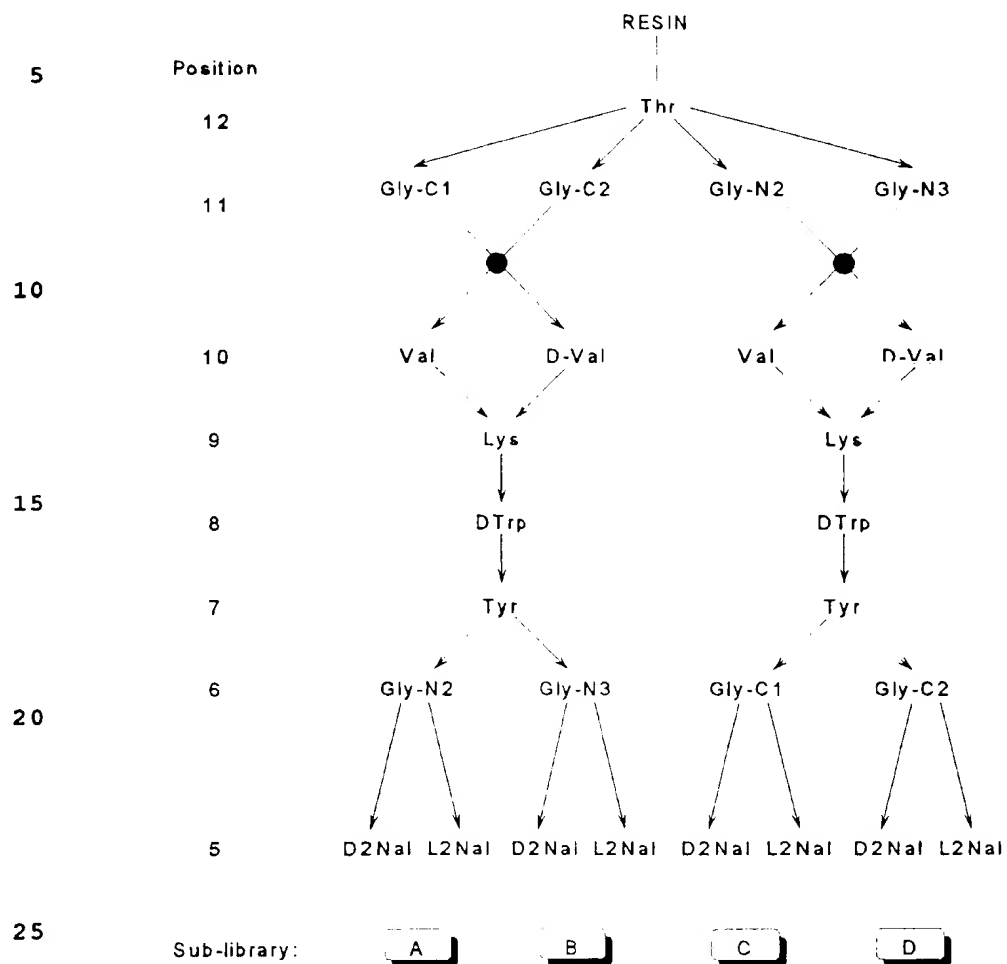
Example 74: YS-SST5 A and B libraries

These libraries were designed for optimization of bridge size and direction between positions 6 and 11 with simultaneous determination of the influence of various Naphtylalanine residues at position 5. The YS-SST-5A library consists of 4 sub-libraries with 16 peptides in each. The YS-SST-5B library, represents a simplified synthetic scheme of sub-libraries C and D, consisting of 8 sub-libraries of 4

YSSA LIBRARY



	A	B	C	D
hSSTR5	200	90	80	80
hSSTR4	400	400	400	400
mSSTR3			80	
mSSTR2				
hSSTR1	20	80	80	80

YS5B LIBRARY

	A1	A2	B1	B2	C1	C2	D1	D2
hSSTR5	700	600	1000	1000	3000	3000	2000	1000
hSSTR4								
mSSTR3								
mSSTR2	3000	2000	1500	1500	1000	2500	1500	800
hSSTR1	400	400	400	400	1000	400	400	400

Example 75: VH-SST6 library

This library, which contains 24 hexapeptides, is described in table 14. Two different Phe-building units were incorporated at position R⁶ with additional diversity at positions R⁷, R⁸ and R⁹. The peptides were cyclized between the backbone of positions R⁶ and R¹⁰. Amino acids at positions 5 and 12 were omitted.

Table 14. The composition of VH-SST6 library

R ⁵	R ⁶	Residues per position					
		R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²
	Phe-C1 Phe-C2	Phe pNO ₂ Phe Phg	DTrp DThi	Lys Orn	Gly-C2	Phe	

15 Example 76: VH-SST7 and VH-SST7A library

These libraries each contain 290304 backbone-cyclic peptides in 45 sub-libraries. The peptides were synthesized on non-cleavable resin (TentaGel-NH₂), yielding bead-attached peptides for screening in solid-phase-assays. The composition of these libraries are described in table VIII. The libraries differ from one another only in that VH-SST7 contains Trp at position 8, whereas VH-SST7A contains D-Trp at the same position.

Table 15. Composition of VH-SST7 library.

Position	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²	
Groups	A	B	C	D	E	F	G	H	
5	1	DPhe	Gly-N2	Phe	Trp or D- Trp	Lys	Thr	Gly- C1	Thr
	2	Phg	Gly-N3	Tyr	Tic	Arg	Val	Gly- C2	Ser
	3	1Nal	Phe-N2	Phg			Ser	Gly- C3	Val
10	4	D1Nal	Phe-N3	pClPhe			Abu	Phe- C1	1Nal
	5	2Nal	Ala-N2	pFPhe				Phe- C2	D1Na 1
	6	D2Nal	Ala-N3	pNO ₂ Phe				Phe- C3	2Nal
15	7	Thi							D2Na 1
	8	DThi							
	9	pClPhe							
	10	pFPhe							
	11	pNO ₂ Phe							
20	12	Des							Total
	Groups	12	6	6	2	2	4	6	7
	Pept. per group	24192	12096	12096	14515 2	14515 2	7257 6	12096	4147 2
									290304

25

The sub-libraries are named for their defined position: A, A, ...A, B... B, ...H...H. For each group, positions other than the defined one, contain mixture of amino acids. In each coupling step, each non-defined position gets a

30 mixture of amino acids (total 1 molar equivalent of amino acids in each step in order to force the completion of each amino acid coupling and to eliminate kinetic effects, yielding non-equal presentation of peptides), that will be present at this position. Identification of the most active

cyclic peptide from the 290304 peptides presented in the library.

Example 77: YS-SST6 library

5 This library comprises 128 backbone-cyclized somatostatin analogs in 8 sub-libraries described in table 16. Two basic cyclizations were used: position 3 to position 7 and position 2 to position 6. Each sub-library differs in the bridge location, bridge type and direction or amino acid
10 at position 1 (Ala or D2Nal).

Table 16. Structure of YS-SST6 sub-libraries.

Sub-library	Residues per position								
	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹
15	A1	Ala	Gly	Gly-C1 Gly-C2	Lys Arg	Asn DAsn	Phe	Gly-N2 Gly-N3	DTrp Lys
	A2	D2Nal	Gly	Gly-C1 Gly-C2	Lys Arg	Asn DAsn	Phe	Gly-N2 Gly-N3	DTrp Lys
	A3	Ala	Gly	Gly-N2 Gly-N3	Lys Arg	Asn DAsn	Phe	Gly-C1 Gly-C2	DTrp Lys
	A4	D2Nal	Gly	Gly-N2 Gly-N3	Lys Arg	Asn DAsn	Phe	Gly-C1 Gly-C2	DTrp Lys
20	B1	Ala	Gly-C1 Gly-C2	Gly	Lys Arg	Asn DAsn	Gly-N2 Gly-N3	Phe	DTrp Lys
	B2	D2Nal	Gly-C1	Gly	Lys Arg	Asn DAsn	Gly-N2 Gly-N3	Phe	DTrp Lys
	B3	Ala	Gly-N2 Gly-N3	Gly	Lys Arg	Asn DAsn	Gly-C1 Gly-C2	Phe	DTrp Lys
25	B4	D2Nal	Gly-N2 Gly-N3	Gly	Lys Arg	Asn DAsn	Gly-C1 Gly-C2	Phe	DTrp Lys

Example 78: IG-SST9 library

In order to more systematically test the necessity of
30 any given frame in the SRIF sequence for biological activity of the analogs, a library of octapeptide analogs that differ from one another in spanning different parts of the SRIF structure was synthesized in parallel. Each octapeptide sub-library is shifted from the next by one residue. Thus, the
35 first sub-library spans residues 2 to 11 of the SRIF sequence.

This library comprises a total of 14 overlapping backbone-cyclized octapeptides with a shift of one residue between each sub-library.

The synthesis is achieved by simultaneous synthesis of
5 the analogs from different starting points, such that the coupling of the building units is performed for all the sub-libraries at the same time. In all these sub-libraries the backbone cyclization is accomplished between one Glycine-C2 unit distal to the N terminal of the peptide sequence and one
10 Glycine N3 unit proximal to the N terminal end of the peptide sequence.

Library IG-SST9 is represented in the following scheme:

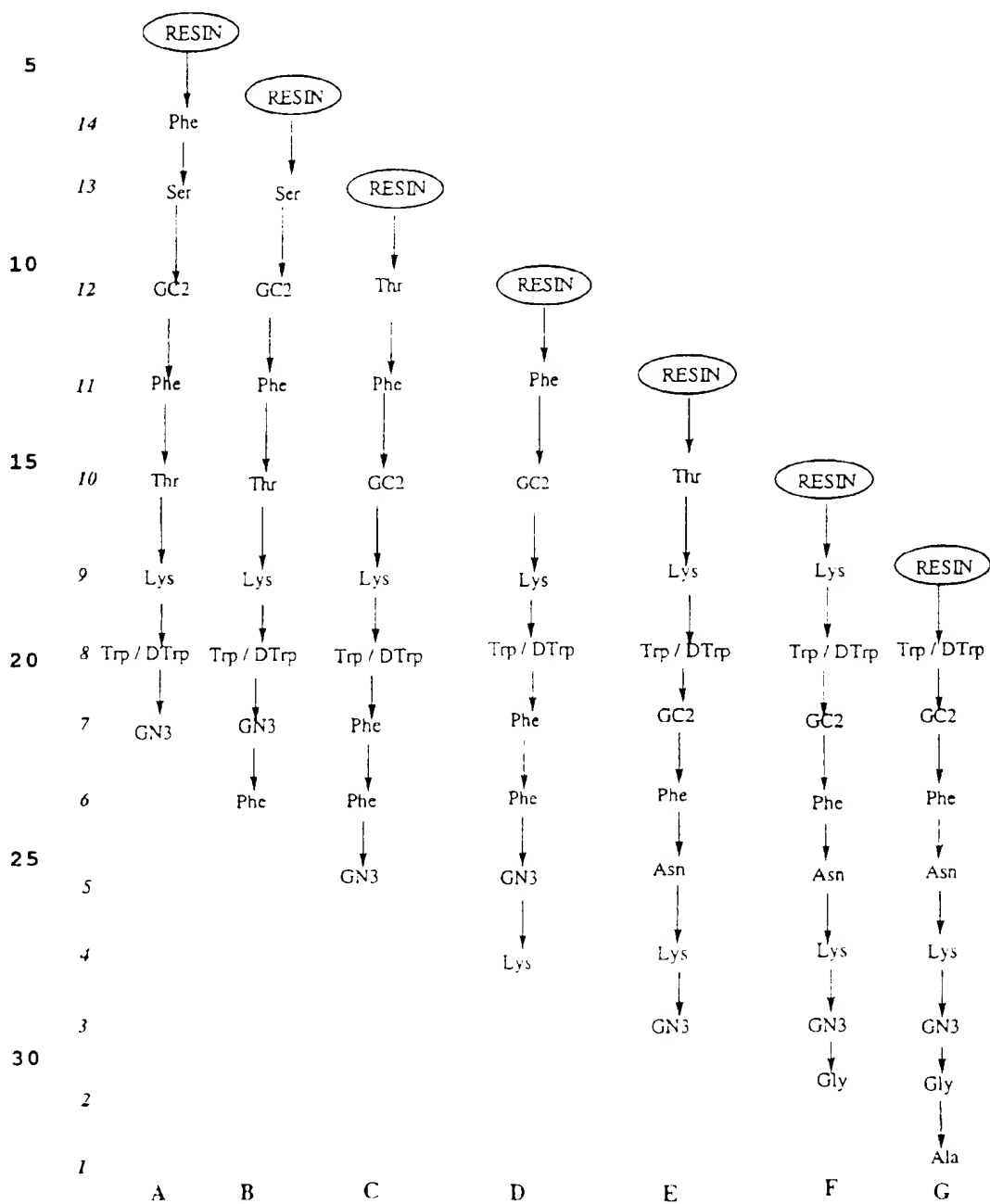
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IG-SST9 Library

Example 79: SST14 library

Different Phenylalanine-building units (PheBU: Phe-N2, Phe-N3, Phe-C2, Phe-C3), are used in this library as bridging arms for the generation of backbone cyclized analogs of SRIF sequence 4-11 (sub-library D in IG-SST9 library). In addition, the non bridging Phe residue (position 6 or 7), is substituted with various Phe and Nal derivatives: DPhe, pNO₂Phe, pClPhe, pFPhe, Phenylglycine (Phg), DPhg, L2Nal, D2Nal. This provides library of 18 groups and 16 analogs per group as described in the following table:

Table 17. The composition of SST14 library

Residues per position							
R ⁴	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹
15	Lys/DLys	Asn	Phe	Phe	Trp/DTrp	Lys	Thr
			PheBU	PheBU			
			(4)	(4)			
			DPhe	DPhe			
			pNO ₂ Phe	pNO ₂ Phe			
20			pClPhe	pClPhe			
			pFPhe	pFPhe			
			Phg	Phg			
			DPhg	DPhg			
			L2Nal	L2Nal			
25			D2Nal	D2Nal			

Example 80: YS-SST7 library

The library contains 48 analogs with the following composition:

30

Table 18. YS-SST7 library

Residues per position							
R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	R ¹²
L2Nal	Gly-N3	pCl-Phe	DTrp	Lys	Ser	Gly-C2	Thr

Example 81. YS-SST10 library.

This library contains 5 sub-libraries with 24 peptides in each as described in the following scheme.

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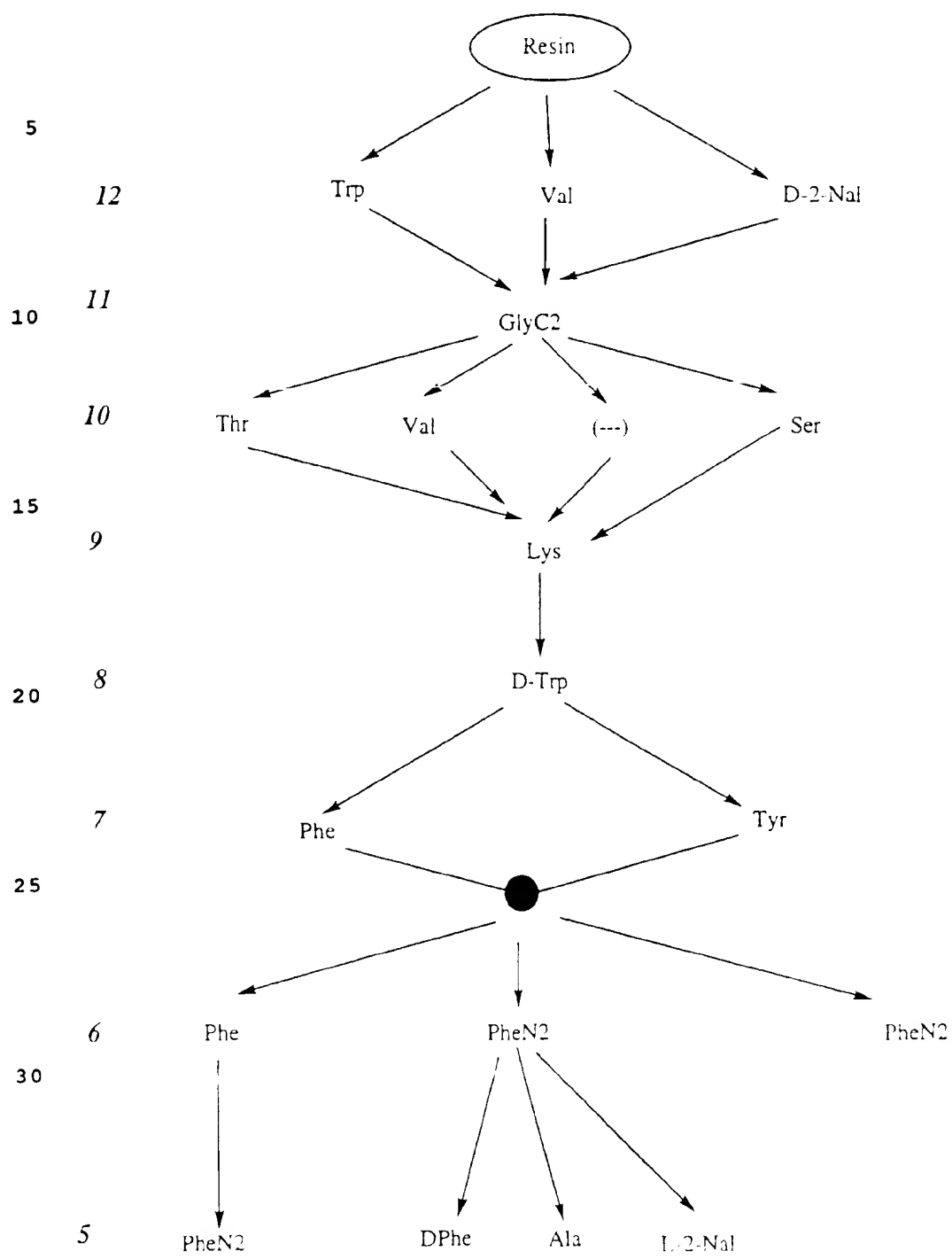
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Example 82. YSS-SST12

The library, described in table 19, represents retro-backbone-cyclized analogs of somatostatin, and is composed of 6 sub-libraries, with 18 analogs in each. The different sub-libraries are defined by the type of bridge (Phe-C2¹¹ to Gly-N3¹⁰ or Phe-N3¹¹ to Gly-C2¹⁰), and residue at position R¹⁰.

Table 19. The composition of YS-SST12 library

10	Sub-library	Residues per position						
		R ¹²	R ¹¹	R ¹⁰	R ⁹	R ⁸	R ⁷	R ⁶
	A		Phe-C2	DVal	DLys	Trp	DPhe	Gly-N3
	B			DThr		DTrp	pNH2Phep	Phe
	C			DGlu			C1DPhe	L2Nal
15	D		Phe-N3	DVal	DLys	Trp	DPhe	Gly-C2
	E			DThr		DTrp	pNH2Phe	L2Nal
	F			DGlu			pC1DPhe	D2Nal

Example 83. YS-SST15 library

This library of heptapeptides consist of 96 analogs in 8 sub-libraries defined by the residues at positions R⁶ and R⁷.

20

Table 20. The composition of YS-SST15 library

25	Sub-library	Sequence per position						
		R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹
	A		Phe-N2	Phe	DTrp	Lys	Gly	Phe-C3
	B		Phe-N3		Trp	DLys		Thr
	C		Lys-N2					Val
	D		Gly-N2					β-Ala
30	E		Phe-N2		DTrp	Lys	Gly	Phe-C3
	F		Phe-N3	Tyr	Trp	DLys		Thr
	G		Lys-N2					Val
	H		Gly-N2					β-Ala

PHYSIOLOGICAL EXAMPLES

Somatostatin analogs according to the present invention were tested for their activity in bioactivity assays *in vitro* and *in vivo*, in comparison to the following: the natural

analog Octreotide; the non cyclized somatostatin derivatives, and/or irrelevant peptides as negative controls.

**Example 84: In vitro radio ligand binding assay for
5 somatostatin.**

The somatostatin analogs were tested for their potency in inhibition of the binding of ^{125}I -Tyr¹¹-SRIF (based on the method described by Raynor et. al., Molecular Pharmacology 43, 838-844, 1993) to membrane preparations expressing the
10 transmembranal somatostatin receptors (SSTR-1,2,3,4 or 5). The receptor preparations used for these tests were either from the cloned receptors selectively and stably expressed in Chinese Hamster Ovary (CHO) cells or from cell lines naturally expressing the SSTRs. Typically, cell membranes
15 were homogenated in Tris buffer in the presence of protease inhibitors and incubated for 30-40 minutes with ^{125}I -Tyr¹¹-SRIF with different concentrations of the tested sample. The binding reactions were filtered, the filters were washed and the bound radioactivity was counted in gamma counter. Non
20 specific binding was defined as the radioactivity remaining bound in the presence of 1 μM unlabeled SRIF-14. In order to validate positive signals of the binding tests, and to eliminate non-specific signals, samples of irrelevant peptides, such as GnRH, that were synthesized and handled
25 using the same procedures, were tested in the same assays as negative control samples. These samples had no binding activity in any of the assays.

**Example 85: Receptor binding specificity of cyclic peptide
30 analogs.**

The various somatostatin receptor subtypes are thought to be involved in different signal transduction pathways. This will have implications in terms of choosing a somatostatin analog which shows specific and selective

Bell (Endocrine Rev. 16, 427-442, 1995), the activities of several receptor subtypes is thought to be as follows:

SSTR-1 and SSTR-2: activation of tyrosine phosphatase which can lead to inhibition of EGF receptor

5 autophosphorylation, a process that is related to the antiproliferative effect of SST.

SSTR-2: inhibition of growth hormone and gastrin release, processes that are relevant to the treatment of acromegaly and anti proliferative effects via growth factors.

10 SSTR-5: inhibition of insulin, lipase, amylase release, activities that are relevant to the inhibition of calcium influx and to anti-proliferative effects of SST.

SSTR-3: involved in angiogenesis.

Binding of representative peptides of Examples 1-50 to
15 different somatostatin receptors was measured *in vitro*, in Chinese Hamster Ovary (CHO) cells expressing the various receptors. An example of the selectivity obtained with the cyclic peptides is presented in Table 21. The IC₅₀ values presented are concentration required to inhibit 50% of
20 radioactive iodinated somatostatin (SRIF-14) binding.

Table 21 Estimated IC₅₀ values (nM) for Inhibition of ¹²⁵I-SRIF-14 binding to cloned SSTRs by selected backbone-cyclized analogs.

25	PTR No.	SSTR-1	SSTR-2	SSTR-3	SSTR-4	human SSTR-5	rat SSTR-5
	3004		> 10000	> 10000			> 2000
	3007		> 10000	> 10000			> 2000
	3010	2500	> 10000	> 1000	> 10000	> 2000	500
	3013		> 10000	> 10000			> 2000
30	3015	2500		> 2000	> 2000	> 2000	1000
	3019		> 10000	> 10000			
	3022					> 2000	
	3025			> 10000	> 2000	> 2000	> 2000
	3034			> 10000	> 2000		> 2000
	3040	100	> 1000	3500	1000	2000	100

The IC_{50} values were calculated by testing the analogs at concentrations of 10^{-6} , 10^{-7} , 10^{-8} M in the radioligand binding assay described in example 84.

The affinities of PTR 3046 (synthetic example no. 42),
5 to the clones SSTRs are shown in figure 1. The unexpected advantages of PTR 3046 over other somatostatin analogs is in its selectivity. The analog binds with high affinity to the human SSTR-5 and much less to the other SSTRs.

Furthermore, the affinity to the rat and the human SSTR5
10 are similar for PTR 3046, thus, drug dosages given in rat models may predict the efficacy in humans. For the sake of comparison the affinities of PTR 3046 are presented in Table 22 together with those obtained for known SST analogs.

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Table 22. Selectivity of PTR 3046 versus known SST analogs to SSTR subtypes.

Analog	IC ₅₀ value (nM)					
	SSTR 1	SSTR 2	SSTR 3	SSTR 4	hSSTR 5	rSSTR 5
SRIF-14*	0.1	0.3	0.1	1.2	0.2	0.3
Octreotide*	>100 0	0.4	150	>100 0	32	0.2
Somatuline (BIM-23014)*	800	2	6	>100 0	14	0.5
RC-160**	200	0.2	0.1	620	21	0.2
BIM-23052*	23	32	0.4	18	4	0.004
PTR 3046	3000	500	500	250	22	30

* Reisine and Bell (1995), Endocrine Reviews 16; 427-442.

** L. Buscail et. al. (1995), PNAS 92; 1580-1584.

Another cyclic peptide analog, PTR 3040, showed an interesting profile of selectivity. This analog shows relatively high affinity for receptor subtype SSTR-1, and very low affinity for the other receptor subtypes. While PTR 3040 showed high binding to the rat SSTR-5, its affinity to the cloned human receptor was significantly lower.

Inhibition of ¹²⁵I-SRIF binding to mouse pituitary AtT20 cells was also tested for various analogs. The results obtained with PTR 3046 and PTR 3040 are presented in Figure 2, compared to SRIF-14. The results represent the percent inhibition per specific concentration of each of the compounds.

Example 86: Receptor binding specificity of libraries of cyclic peptide analogs.

Binding of representative sub-libraries of peptides of Examples 68-83 to different somatostatin receptors was measured in vitro, in Chinese Hamster Ovary (CHO) cells

presented in Table 23. The values presented are percent inhibition of radioactive iodinated somatostatin (SRIF-14) binding.

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Table 23. Estimated IC_{50} values (nM) for Inhibition of ^{125}I -SRIF-14 binding to cloned SSTRs by selected backbone-cyclized sub-libraries.

5	Sub-library	Pep. *	SSTR-1	SSTR-2	SSTR-3	SSTR-4	human SSTR-5	rat SSTR-5
	VH1-A	8						8000
	VH1-B	8						6400
	VH1-C	8						7200
	VH1-D	8						1600
	VH1-E	8	1600		>2000	8000	6400	640
10	YS1-A1-2	4				>5000		3200
	YS1-A3-4	4				>5000		800
	YS1-B1-2	4	360	6000	3600	600	1600	200
	YS1-B1-4	4				>5000		8000
	YS2-A	12				3600		100
	YS2-B	12				3600		120
	YS2-C	12	110	1200	300	1000	100	25
	YS2-D	12	960	300	4800	1800	3000	25
15	YS4	24	3500			2400	2400	2000
	VH6	24						7200
	YS5A-A	16	320			6400	3200	1300
	YS5A-B	16	1300			6400	1400	
	YS5A-C	16	1300		1300	6400	1300	
	YS5A-D	16	1300			6400	1300	
20	YS5B-A1	4	400	3000			700	
	YS5B-A2	4	400	2000			600	
	YS5B-B1	4	400	1500			1000	
	YS5B-B2	4	400	1500			1000	
	YS5B-C1	4	1000	1000			3000	
	YS5B-C2	4	400	2500			3000	
	YS5B-D1	4	400	1500			2000	
	YS5B-D2	4	400	800			1000	

25 * Number of different peptides in each sub-library.

The estimated IC_{50} are calculated based on the concentration of total peptides in each sub-library mixture. However, each sub-library is composed of different peptides that might have different activity. The activity of the best peptide in any given sub-library might therefore be higher (i.e., lower IC_{50} value), to the extent of the given value divided by the number of peptides per sub-library. In addition, the activity values of purified individual peptides might be better due to existence of salts and impurities.

In vivo examples:

Selected analogs were tested in-vivo, for the inhibition of growth-hormone release, amylase, lipase, gastrin, insulin, cholecystokinin (CCK), VIP and glucagon secretion in animals.

- 5 Growth hormone (GH) release is related to acromegaly. Gastrin secretion is related to gastrinoma (Zollinger-ellison syndrome) and ulcer. Insulin release is related to insulinoma, hyperinsulinoma, obesity and NIDDM. Glucagon release is related to hyperglycemia, NIDDM and glucagonemia.
- 10 Amylase and lipase release are related to acute and chronic pancreatitis, enterocutaneous and pancreatic fistulas and pancreatic surgery. VIP release is related to VIPoma and secretory diarrhea. In addition, the antiproliferative effects of somatostatin can be direct or indirect through GH-
- 15 IGF or CCK release.

Example 87: Somatostatin biological activity assays (In vivo assays).

- The *in vivo* biological effects of SST analogs on growth
- 20 hormone, insulin and glucagon release is tested by measuring the levels of these hormones using commercially available RIA test kits. Pharmacological effects of SST in patients with neuroendocrine tumors of the gut will require determination of 5-hydroxyindole acetic acid (for carcinoid tumors) and VIP
- 25 (for VIPoma). *In vivo* visualization of SST receptor-positive tumors is performed as described by Lambert et al. (New England J. Med., 323:1246-1249 1990), following i.v. administration of radio-iodinated SST analogs.

30 Example 88: Resistance to biodegradation of SST analogs.

- The *in vitro* biostability of a SST cyclic peptide analog, PTR 3002, was measured in human serum, and was compared to the same sequence in a non-cyclic peptide analog (PTR 3001), to Octreotide (Sandostatin™), and to native
- 35

structure, and is much more stable than SRIF. The assay was based on HPLC determination of peptide degradation as a function of time in serum at 37°C.

5 Example 89: Inhibition of growth hormone release by SST analogs.

In vivo determination of the pharmacodynamic properties of cyclic peptide SST analogs was carried out in rats, according to known procedures. Inhibition of Growth Hormone (GH) release as a result of peptide administration was measured in Sprague-Dawley male rats. The SST cyclic peptide analog activity was compared in this study to SRIF or to Octreotide using 4 rats in each group. Time course profiles for GH release under constant experimental conditions were measured.

Methods

Adult male Sprague-Dawley rats, specific pathogen free (SPF), weighing 200-350 g, were maintained on a constant light-dark cycle (light from 8:00 to 20:00 h), temperature (21 ± 3°C), and relative humidity (55 ± 10%). Laboratory chow and tap water were available ad libitum. On the day of the experiment, rats were anesthetized with pentobarbitone (50 mg/kg). Rats anesthetized with pentobarbitone exhibit low somatostatin levels in portal blood vessels (Plotsky, P.M., Science, 230, 461-463, 1985). A single blood sample (0.6 ml) was taken from the exposed cannulated jugular vein for the determination of the basal GH levels (-15 min.). Immediately thereafter the appropriate peptide treatment was administered. The animals received 10 mg/kg of either native somatostatin (SRIF), the synthetic analog Octreotide (Sandostatin), or the cyclic peptide analog. A physiological saline solution (0.9% NaCl) was administered as a control. All peptides were administered subcutaneously in a final volume of 0.2 ml. Further sampling was carried out at 15,

units per ml of blood) and centrifuged immediately. Plasma was separated and kept frozen at -20 C until assayed.

Rat growth hormone (rGH) [125 I] levels were determined by means of a radioimmunoassay kit (Amersham). The standard in
5 this kit has been calibrated against a reference standard preparation (NIH-RP2) obtained from the National Institute of Diabetes and Digestive and Kidney Diseases. All samples were measured in duplicate.

The results of these experiments show that Octreotide
10 significantly inhibits the release of growth hormone whereas the cyclic analog PTR 3046, that does not bind the SSTR-2, is not significantly different from saline in this test, as expected for a somatostatin analog which is selective for the SSTR-5 receptor subtype.

15 The data of these studies are summarized in Figure 3.

Example 90: Lack of toxicity of cyclized peptide analogs.

PTR 3007 at a dose of 1.5 mg/kg was well tolerated after single intraperitoneal application. PTR 3013 was not toxic
20 to the rats even with doses of 4 mg/kg. These two doses are several orders of magnitude higher than those needed to elicit the desired endocrine effect. The peptides dissolved in saline produced no untoward side effects on the central nervous system, cardiovascular system, body temperature, nor
25 on the periphery of the animals. Rats were observed for 4 hours post administration of the peptides. PTR 3007 and 3013 produced no respiratory disturbances, did not result in the appearance of stereotyped behavior, or produce any changes in muscle tone. After 3 hours, postmortem
30 examination did not detect any abnormality in the liver, kidneys, arteries and veins, gastrointestinal tract, lungs, genital system, nor the spleen.

Example 91. The *in vivo* effect of somatostatin analogs on
35 glucose induced insulin release in rat

to evaluate the effect of the backbone cyclic analog PTR 3046 on the postprandial secretion of insulin.

Experimental:

5 Adult male Wister rats, specific pathogen free (SPF) weighing 200-220 g were used. The animals were maintained on a constant light- dark cycle (light from 8.00 to 20.00 h), Temp. (21°C), and humidity (55%). The animals were allowed free access to food and water until 18-20 hours before the
10 experiment, when all food (but not water) was withdrawn. The animals were housed in plastic cages with wide mesh wire bottoms to prevent coprophagia (feeding on excrement). On the day of the experiment, rats were anesthetized with pentobarbitone (60 mg/kg IP). 15 minutes after pentobarbital
15 administration a catheter was inserted into the right external jugular vein to allow sampling of blood. Body temperature was monitored using a rectal temperature digital thermometer, and maintained at a constant level (37-37.5°C) by heated blanket placed beneath the rat in addition to two
20 100W bulbs illuminating the operation table from a distance of 50 cm. After cannulation, a 0.7 ml sample of blood was drawn from the jugular vein, and transferred into tubes into which 20 IU heparin solution 5000 IU/ml was previously prepared. This blood sample was collected for the
25 determination of the basal insulin levels. Immediately thereafter the appropriate peptide pre-treatment was administered.

The animals received the following peptides: The synthetic analog Octreotide at 10 µg/kg (n=15), the backbone
30 cyclic peptide PTR 3046 at 7 µg/kg (n=18). Saline (0.9% NaCl) 0.2 ml was administered as a control (n=16). All peptides were administered subcutaneously in a final volume of 0.2 ml. 10 minutes after drug administration the next blood sample was drawn, immediately after that a glucose

Further sampling was carried out at 2 and 5 min. post glucose administration.

Immediately after the collection of each blood sample an appropriate volume (0.7 ml) of saline was administered IV.

5 Blood samples were collected into tubes containing heparin (15 units per ml of a blood) and centrifuged (1500 g) immediately and plasma separated and kept frozen at -20°C (until assayed).

10 Rat insulin (rIns) [125 I] levels were determined by means of rat insulin RIA kit. This kit utilizes an antibody made specifically against rat insulin (Linco). The sensitivity of the kit was 0.1 ng/ml. All samples were measured in duplicate.

15 Results:

Administration of 7 µg/kg of PTR 3046 led to a significant ($p < 0.05$) decrease (43%) in insulin release in comparison to untreated control rats. The decrease of the insulin levels in Octreotide pretreated rats were not
20 statistically significant in this experiment.

These results demonstrated that the backbone cyclic peptide PTR 3046 is a potent inhibitor of postprandial insulin release in vivo. Based on the present study and the manufacturer's data reported for Octreotide (ED 50 for
25 insulin release of 26 µg/kg) it is concluded that in vivo PTR 3046 is 4 times more potent than Octreotide on insulin release.

Hyperinsulinemia is one of the etiologic factors associated with the pathology of obesity and the early stage
30 of noninsulin dependent diabetes mellitus (NIDDM).

Therefore, the potential application of PTR 3046 as an anti secretagogue on insulin release should be considered in view of the high inhibition of insulin secretion with this novel SST analog.

35

Example 92: Effect of somatostatin analogs on bombesin stimulated plasma amylase and lipase release.

Inhibition of either insulin or amylase release has been reported (Reisine and Bell, *ibid.*) to be highly correlated with affinity to the SST receptor subtype SSTR-5, while inhibition of pituitary growth hormone (GH) release, pancreatic glucagon, and gastric acid secretion were SSTR-2 mediated. Binding affinity data presented above (Example 85), demonstrated the selectivity of the cyclic heptapeptide PTR 3046 to hSSTR-5 (affinity 20 nM) in comparison to its low affinity (>1000 nM) to SSTR-1, 2, 3 and 4. Based on these results the preliminary evaluation of the physiological activity of PTR 3046 was evaluated using the *in vivo* model of bombesin stimulated lipase and amylase release. The dose response of PTR 3046 in terms of inhibition of amylase and lipase release in rats was measured following administration of the peptide analog at three different dosages i.e., 3 $\mu\text{g/kg}$, 12.5 $\mu\text{g/kg}$ and 25 $\mu\text{g/kg}$. These doses were compared to the synthetic analog SMS-201 995 administered at a dose of 10 $\mu\text{g/kg}$.

Methods

Male Wister rats (weighing 200-220 g) were anaesthetized with pentobarbital (60 mg/kg) by the intraperitoneal (IP) route. The animals were allowed free access to food and water until 18 h before experiment, when all food (but not the water) was withdrawn. Furthermore the animals were housed in cages with wide mesh wire bottoms to prevent coprophagia (feeding on excrement).

Collection of samples

After cannulation, a 0.7 ml sample of blood was drawn from the jugular vein, and transferred into an Eppendorf tube containing 20 IU heparin solution 5000 IU/ml. One minute

0.2 ml of 0.9% NaCl. At time 0 (5 min. after collection of baseline blood sample-1) a bombesin infusion (50 nmol/kg/hr) was started in all animals. Additional blood samples were collected at constant time intervals of 60, 90 and 120 min. 5 during bombesin infusion.

Treatment of samples

Blood samples were collected into ice-cold tubes containing heparin (20 IU/ml) and were centrifuged (1500 g x 10 10 min.). Plasma samples were frozen and kept at -20°C until assayed for amylase and lipase.

Analytical- assays

Amylase levels were measured in plasma with the commercial 15 (Raichem™) Amylase reagent.

Lipase levels were measured in plasma with a commercial kit (Randox™).

20 Results

Prevention of bombesin- stimulated amylase secretion by SST analogs:

Control (saline pretreatment):

25 Bombesin I.V. infusion at a dose of 50 nmol/kg/h resulted in a time dependent increase of plasma amylase (by 10 fold above basal at 60 and 90 min.), and lipase (by 14 fold above basal at 60 and 90 min.).

30 PTR 3046:

Pretreatment with PTR 3046 resulted in significant dose dependent inhibition of bombesin induced release of plasma amylase in comparison to control rats. Inhibition was 31% at 90 min. and 23% at 120 min. for 3 µg/kg, and 60% at 90 min.

with Octreotide at 10 $\mu\text{g/kg}$ resulted in a significant inhibition 23% at these time points.

Prevention of bombesin-stimulated lipase secretion by SST
5 analogues:

PTR 3046:

Pretreatment with PTR 3046 resulted in significant dose dependent inhibition of bombesin induced release of plasma
10 amylase in comparison to control rats. Inhibition was 33% at 90 min. and 26% at 120 min. for 3 $\mu\text{g/kg}$, 30% at 90 min. and 25% at 120 min. for 12.5 $\mu\text{g/kg}$, and 51% at 90 and 35% at 120 min. for the dose of 25 $\mu\text{g/kg}$. While pretreatment with
15 SMS at 10 $\mu\text{g/kg}$ resulted in a significant inhibition of 27% and 30% at 90 and 120 min. respectively.

Similar results were obtained with PTR 3010 (data not shown).

These results demonstrate that PTR 3046 is a selective
20 analog for SSTR-5 as shown in preliminary binding assays and physiological model of enzymes and endocrine secretion.

Bombesin induced pancreatic and gastric secretion is a common *in vivo* model for the evaluation of anti secretory effect of bombesin antagonist, bombesin antibodies, and
25 somatostatin analogs.

The putative role of bombesin in several lung and gut diseases in humans suggest that drugs that inhibit the secretory effect of bombesin, like PTR 3010, and PTR 3046 could be used as pharmacotherapy for various therapeutic targets associated with bombesin, including: secretory
30 diseases such as pancreatitis, rhinitis and gastrinoma; neurocrine cell hyperplasia diseases such as bronchopulmonary dysplasia, cystic fibrosis, and chronic bronchitis and emphysema; proliferative diseases such as prostate hypertrophy, prostate cancer, pancreatic cancer and stomach
35

Furthermore, portal hypertension, GI bleeding, and colorectal carcinoma could be potential applications as well.

Example 93. Duodenal-pancreatic Perfusion

5 A limitation with the Bombesin or the Caerulein models was that, both tests were based on the indirect detection of levels of enzymes in the serum. Because amylase can be released from saliva and other sites in the GI tract, there was a need for a direct measurement of effect on
10 the pancreatic release of enzymes. To that end, the perfusion model was developed in the rat. The results (Fig. 5) indicate that PTR 3046 and Sandostatin (administered by IV infusion), both inhibit the pancreatic release of enzymes, as detected directly in the duodenal
15 perfusate.

The duodenal segment which drains the pancreatic release was dissociated proximally from the stomach and distally from the jejunum. The segment was perfused with physiological saline, perfusate was collected at 15 min. time
20 intervals. Stimulation of pancreatic release was done by IV infusion of Bombesin or Caerulein, 1 nMole/kg/h or 4 nMole/kg/h respectively. During perfusion (after the enzyme levels reached plateau levels) drugs were infused
25 IV for another 2 h. Pancreatic enzyme levels were measured in the perfusate samples. Data are presented as percentage of average enzyme levels from bombesin induced plateau levels.

30

Example 94. Antiproliferative activity

Based on the inhibition of exocrine release induced by caerulein (a CCK analog), and because CCK is a potent
35 growth factor in the GI tract.

A significant antiproliferative effect of PTR 3046 was found on the human pancreatic cell line, MiaPaca-2 (Figure 6).

Cells were grown in DMEM culture media supplemented with 5 10% FCS. Cells allowed to attached after 24 h to the plate. Drugs were added to the culture media in concentration range of 10^{-6} M- 10^{-11} M. Proliferation of cells was evaluated by MTT assay at 24, 48, and 72 hours following drug application.

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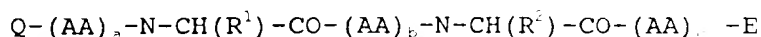
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THE CLAIMS

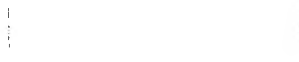
What is claimed is:

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1. A backbone cyclized somatostatin analog having the general Formula (I):



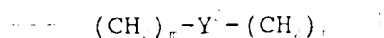
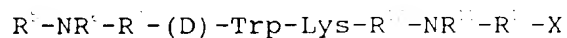
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Formula (I)

wherein: a - c each independently designates an integer
 15 from 1 to 8 or zero; (AA) designates an amino acid residue wherein the amino acid residues in each chain may be the same or different; Q represents H or an acyl group; E represents a hydroxyl group, a carboxyl protecting group or an amino group, or the terminal carboxyl
 20 group can be reduced to CH_2-OH ; R^1 and R^2 each designates an amino acid side-chain optionally bound with a specific protecting group; and the line designates a bridging group of the Formula: $-(CH_2)_x-M-(CH_2)_y-$; M is selected from the group consisting of amide, thioether, thioester and
 25 disulfide; and x and y each independently designates an integer from 1 to 10.

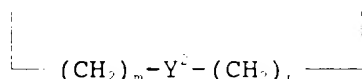
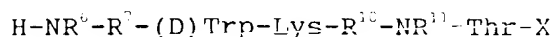
2. The backbone cyclized somatostatin analog of Claim 1
 30 having the general Formula (Va):



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wherein m and n are 1 to 5; X designates a carboxy terminal amide or alcohol; R is absent or is Gly, (D)- or (L)-Ala, Phe, Nal and β -Asp(Ind); R and R' are independently Gly or (D)- or (L)-Phe; R' is Phe or Tyr; R¹⁰ is absent or is Gly, Abu, Thr or Val; R¹¹ is absent or is Val, Thr or Nal, and Y¹ is selected from the group consisting of amide, thioether, thioester and disulfide.

3. The backbone cyclized peptide analog of Claim 2
10 having the general Formula (Vb):

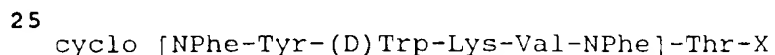


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Figure (Vb)

wherein m and n are 1 to 5; X designates a carboxy terminal amide or alcohol; R¹ and R¹¹ are independently Gly or (D)- or (L)-Phe; R¹⁰ is Phe or Tyr; R¹² is absent or is
20 Gly, Abu, Thr or Val; and Y¹ is selected from the group consisting of amide, thioether, thioester or disulfide.

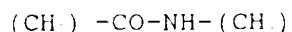
4. The backbone cyclized somatostatin analog of claim 3
having the structure:



wherein X denotes a carboxy terminal acid, amide, ester or alcohol.

30

5. The backbone cyclized somatostatin analog of claim 4
having the structure



35

wherein X denotes a carboxy terminal acid, amide, ester or alcohol.

6. The backbone cyclized somatostatin analog of claim 3
5 having the structure:

cyclo [NPhe-Phe-(D)Trp-Lys-Thr-NPhe]-Val-X

wherein X denotes a carboxy terminal acid, amide, ester or alcohol.

10

7. The backbone cyclized somatostatin analog of claim 6
having the structure

$(CH_2)_2-CO-HN-(CH_3)_2$

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N-Phe-Phe-(D)Trp-Lys-Thr-NPhe-Val-X

wherein X denotes a carboxy terminal acid, amide, ester or alcohol.

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8. The backbone cyclized somatostatin analog of Claim 1
having the general Formula (XI):

25 Formula XI

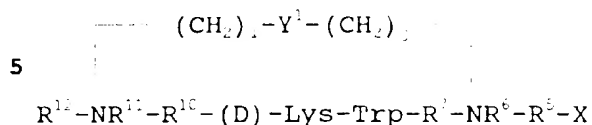
$(CH_2)_2-Y-(CH_2)_2$

$NR^i-R^j-(D)Trp-Lys-R^k-NR^{10}-R^{11}-X$

30

wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R^i is (D)Phe or (L)Phe, Ala or Lys; R^j is absent or is Phe; R^k is Tyr or Phe; R^{10} is absent or Thr, Val, Ser or Abu, R^{11} is Phe, Gly or Ala;
35 R^{12} is Trp, Thr, Val, 2-Nal or (D)2-Nal and Y is

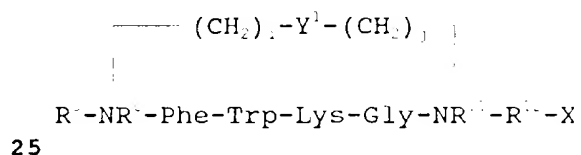
9. The backbone cyclized somatostatin analog of Claim 1 having the general Formula (XII):



Formula XII

10 wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R⁵ is Phe, (L)2-Naphtylalanine or (D)2-Naphtylalanine; R⁶ is Phe, Gly or Ala; R⁷ is (D)Phe, pCl(D)Phe, pNH₂Phe or (D)Tyr; R¹⁰ is (D)Thr, (D)Val, (D)Ala, (D)Leu or (D)Glu; R¹¹ is Phe, Gly
15 or Ala; R¹² is absent or is Thr or Val, and Y¹ is selected from the group consisting of amide, thioether, thioester and disulfide.

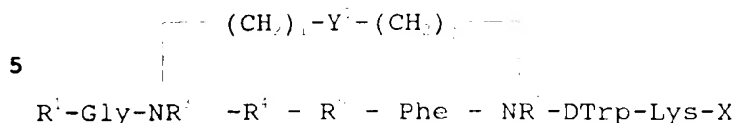
10. The backbone cyclized somatostatin analog of Claim 1
20 having the general Formula (XIII):



Formula XIII

wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R⁷ is absent or (D)Phe
30 or 2-Nal; R⁶ is Phe, Gly, Lys or Ala; R⁸ is (D)Phe, pCl(D)Phe, pNH₂Phe or (D)Tyr; R¹¹ is (D)Thr, (D)Val, (D)Ala, (D)Leu or (D)Glu; R¹² is Phe, Gly or Ala; R¹³ is Thr, Val, Ala, 3-Ala, (L)2-Naphtylalanine or (D)2-Naphtylalanine, and Y¹ is selected from the group
35 consisting of amide, thioether, thioester and disulfide.

11. The backbone cyclized somatostatin analog of Claim 1 having the general Formula (XIV):



Formula XIV

10 wherein i and j are independently 1 to 5; X designates a carboxy terminal amide or alcohol; R¹ is Ala or (D)2-Naphtylalanine; R² is Lys or Arg; R⁵ is (L)Asn or (D)Asn; R⁷ is Phe, Gly, Ala or Lys, and Y is selected from the group consisting of amide, thioether, thioester and disulfide.

12. A pharmaceutical composition comprising the somatostatin analog of any one of claims 1-11 and a pharmaceutically acceptable carrier or diluent.

20 13. The pharmaceutical composition of claim 12 wherein the somatostatin analog is cyclo [NPhe-Tyr-(D)Trp-Lys-Val-NPhe]-Thr-X wherein X is denotes a carboxy terminal acid, amide, ester or alcohol.

25 14. The pharmaceutical composition of claim 13 in unit dosage form.

15. The pharmaceutical composition of claim 12 wherein the somatostatin analog is
30 cyclo [NPhe-Phe-(D)Trp-Lys-Thr-NPhe]-Val-X wherein X denotes a carboxy terminal acid, amide, ester or alcohol.

16. The pharmaceutical composition of claim 15 in unit
35

17. A method of treating endocrine disorders, neoplasms or metabolic disorders which comprises administering to an individual in need thereof a therapeutically effective amount of the somatostatin analog of any one of claims 1-5 11.

18. Use of the somatostatin analog of any one of claims 1-11 in the manufacture of a medicament for the treatment of endocrine disorders, neoplasms or metabolic disorders.

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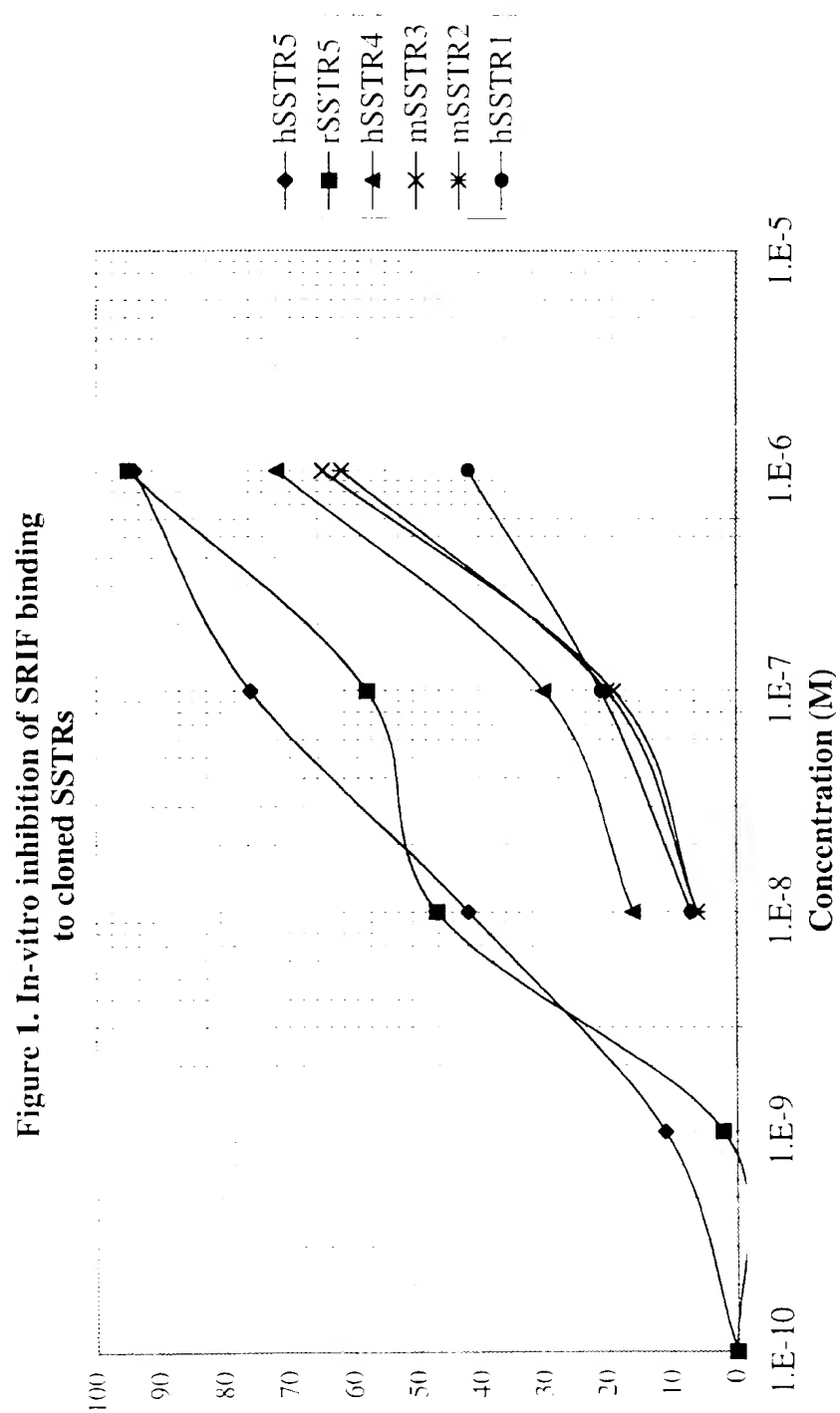
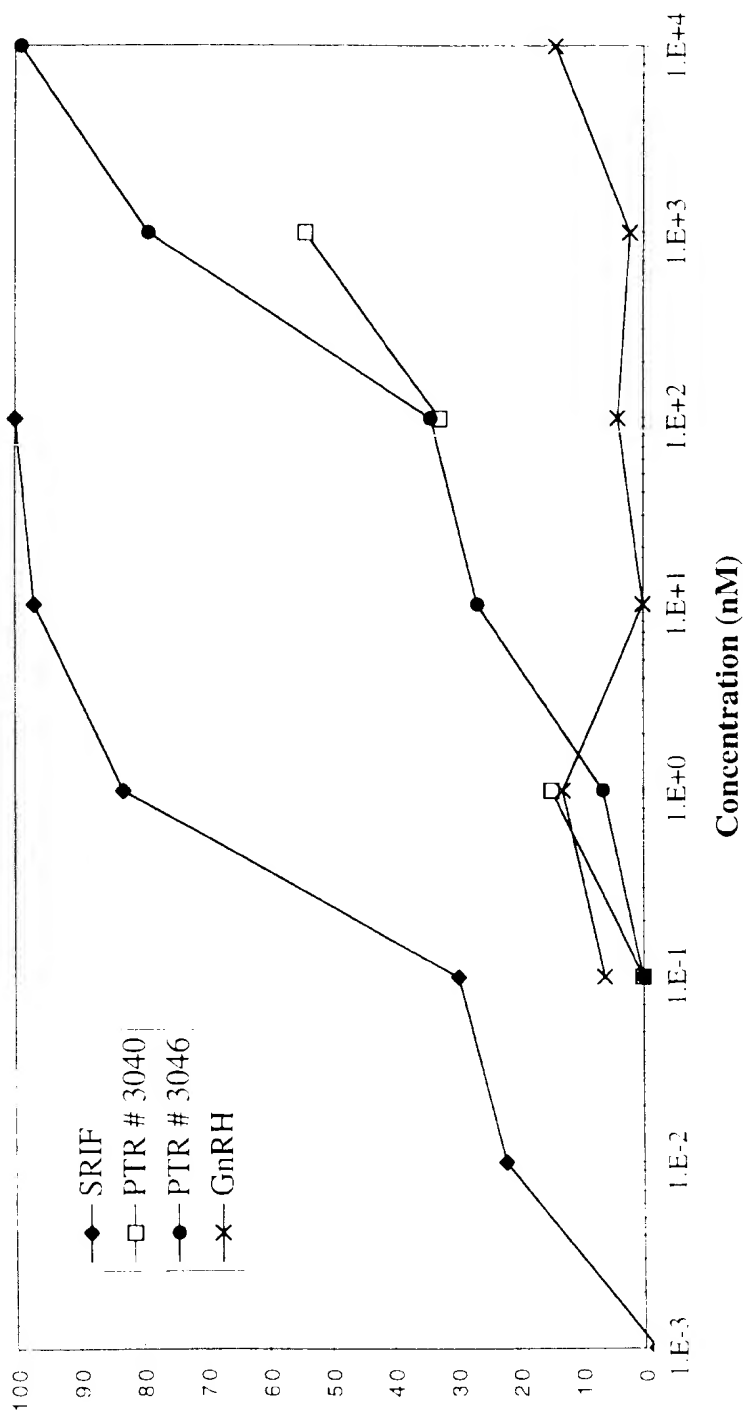


Figure 2. Inhibition of 125 I-SRIF binding to AtT20 cells



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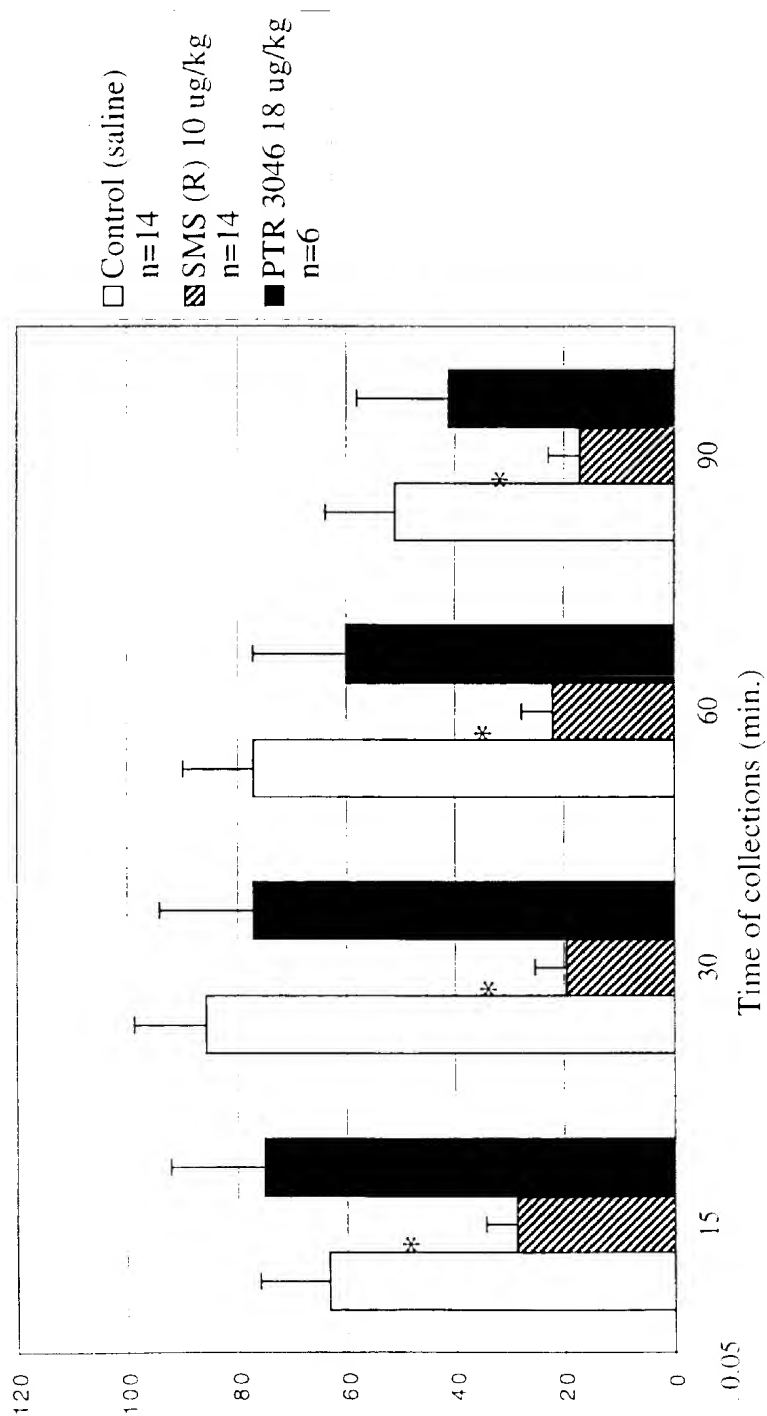
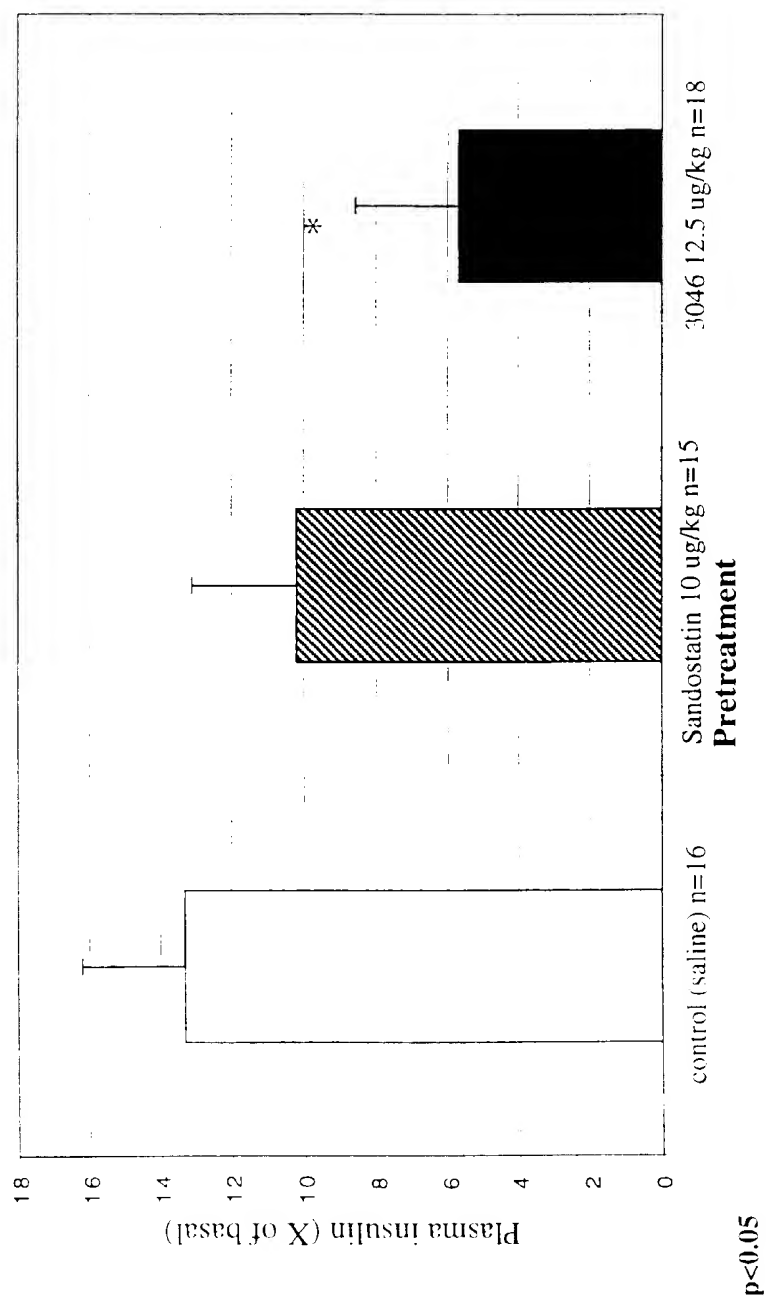
Figure 3. The *in vivo* effect of SST analogs on GH release in rats

Figure 4. The in vivo effect of SST analogs on glucose induced insulin release in rats



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Figure 5. Pancreatic exocrine release in duodenal perfusion following bombesin induction

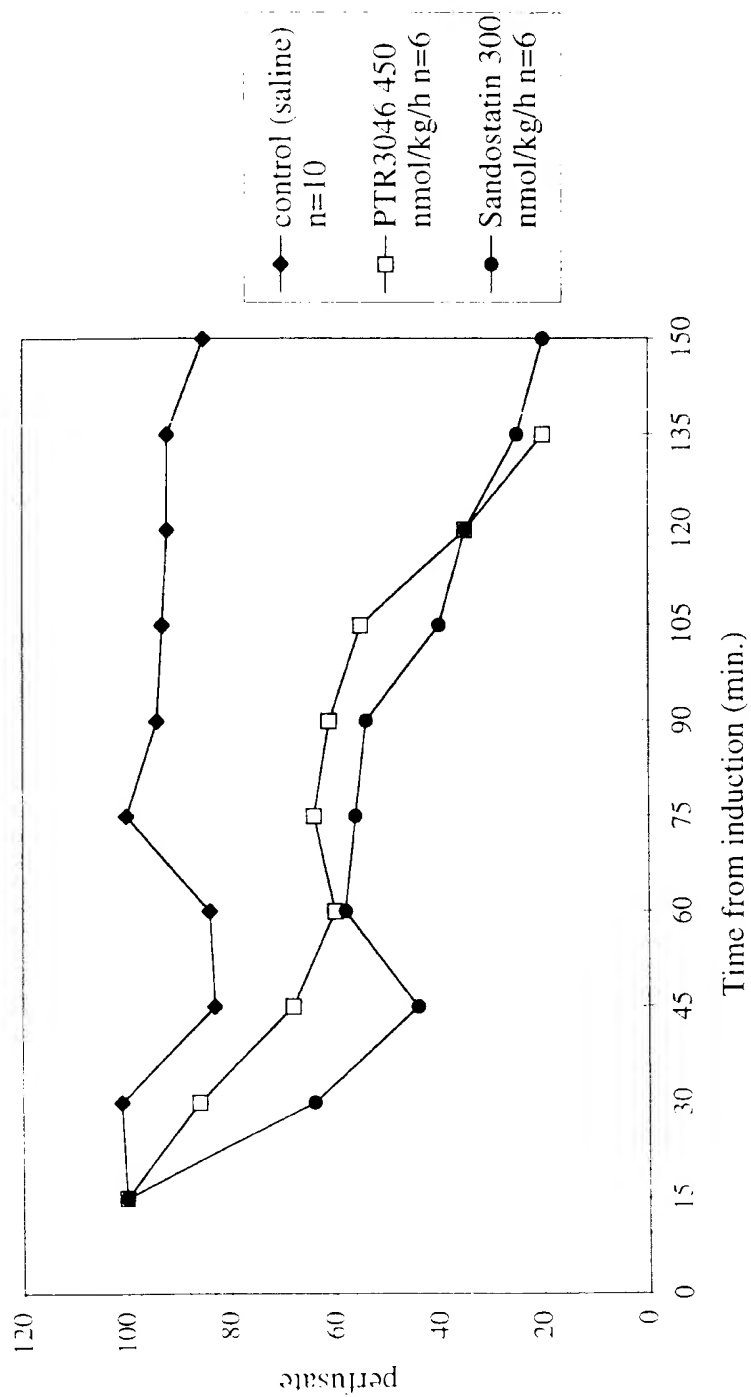
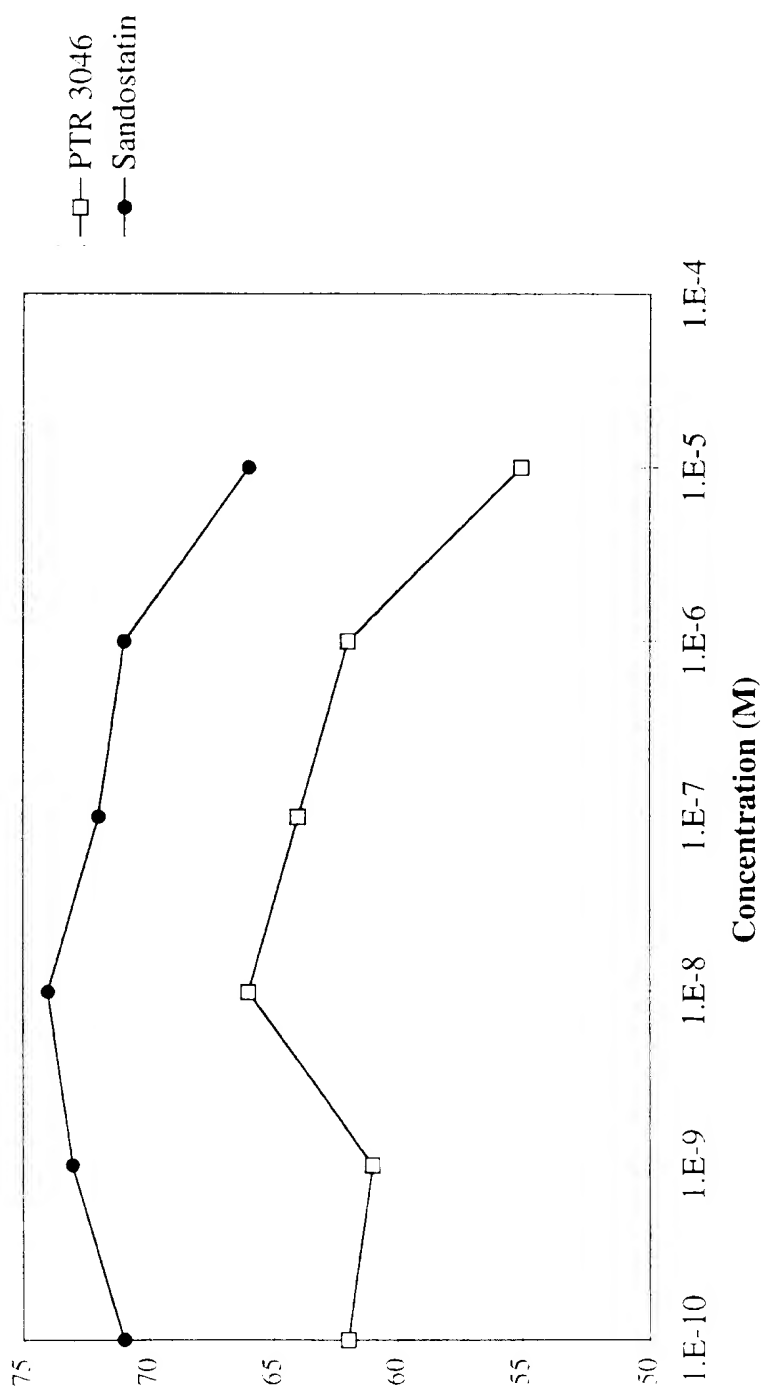


Figure 6. In-vitro antiproliferative effect of PTR3046 on MiaPaca-2 cells



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL97/00261

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 7/50; A61K 38/04, 38/12

US CL : 530/311, 317, 328; 514/11, 16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/311, 317, 328; 514/11, 16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

search terms: somatostatin, backbone cyclization, monocyclic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y — A	EP 0 564 739 A2 (YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM) 13 October 1993 (13.10.93), see entire document.	1-18 ---- 1-18
Y	GILON et al. Backbone Cyclization: A New Method For Conferring Conformational Constraint on Peptides. Biopolymers. 1991, Vol. 31, pages 745-750, see entire document.	1-11
A	BYK et al. Building Units for N-Backbone Cyclic Peptides. 1. Synthesis of Protected N-(ω -Aminoalkylene)amino Acids and Their Incorporation into Dipeptide Units. Journal of Organic Chemistry. 1992, Vol. 57, No. 21, pages 5687-5692.	1-11

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date (if any)	

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Telephone No. (703) 305-0196

International application No.
PCT/IL97/00261

International application No.
PCT/IL97/00261

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y -- A	EP 0 395 417 B1 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 08 February 1995 (08.02.95), see entire document.	1-18 ---- 1-18
A	US 4,011,182 (SARANTAKIS) 08 March 1977, see entire document.	1-18
A	RUDINGER, J. 'Characteristics of the amino acids as components of a peptide hormone sequence' In: Peptide Hormones. Edited by J.A.Parsons. Baltimore: University Park Press, 1976 pages 1-7.	1-18